(72) Inventors: FERRAN, Christiane; 92 Landseer Street, West Roxbury, MA 02132 (US). ARVELO, Maria, B.; 22 Berry Street, Quincy, MA 02169 (US).

(54) Title: USE OF PRO-APOPTOTIC FACTORS IN TREATMENT OF ATHEROSCLEROSIS

(57) Abstract: The present invention features a novel method of treating vascular disease that involves modifying smooth muscle cells to express a gene encoding a protein having both anti-inflammatory and pro-apoptotic activity. Preferably, the protein of the invention also has an anti-proliferative effect in smooth muscle cells. In general, the method is useful in preparing vascularized organs and vessels for transplant into a patient. Alternatively, the present invention can be applied to treat atherosclerotic lesions in damaged vessels.

USE OF PRO-APOPTOTIC FACTORS IN TREATMENT OF ATHEROSCLEROSIS

5   ## Statement as to Federally Sponsored Research

The present research was supported by a grant from the National Institutes of Health (NIH) (Grant R01 Number HL57791-02).

Field of the Invention

The present invention relates to the field of vascular disease.

10   ## Background of the Invention

Vascular disease is the most common cause of morbidity and mortality in the western world, surpassing any other single degenerative disease. The fundamental pathology of vascular disease is an abnormal accumulation of cells within the subintimal space below the surface of the

15   endothelial cell lining, resulting in a decrease in lumen size and tissue perfusion. This accumulation is due to the proliferation and/or migration of smooth muscle cells, and/or inflammatory cells into the intimal layer of a vessel, resulting in restricted blood flow through that vessel, i.e. neointimal occlusive lesions.

20   Current efforts aimed at preventing and treating vascular disease are directed at developing improved lipid-lowering agents (e.g., for treatment of atherosclerosis) and immunosuppressive regimens. For example, lipid lowering agents are currently used in the treatment of atherosclerosis. Alternatively, immunosuppressive regimes are used in

25   allotransplantation for prevention of transplant-associated vasculopathy

(TAV), also referred to as transplant arteriosclerosis. TAV is closely related to atherosclerosis and remains the major barrier to successful transplantation (Brooks-Wilson et al., *Nature Genetics*, (1999) 22:327-335; Kirk et al., *Nature Medicine* (1999) pages 686-693; Hancock et al.,

5   *Nature Medicine*, (1998) 4:1392-1396). Alternatively, surgical strategies have been developed that are aimed at bypassing the obstruction with venous conduits, or stretching the vessel to create a larger lumen by performing balloon angioplasty.

Despite the substantial benefit attributable to the use of current

10   cholesterol lowering and immunosuppressive drug therapies, these treatments do not achieve an acute reduction in vascular lesion size. Furthermore, bypass surgery may accelerate progressive lesion stenosis, and interventions such as balloon angioplasty often result in the development of restenosis. There exists the need of a strategy for

15   preventing and treating vascular disease.

The role of the protein A20 has been studied extensively in endothelial cells and suggests that A20 plays an anti-apoptotic role in response to inflammatory stimulus.

Summary of the Invention

20   The present invention is based on the finding that A20 is a pro-apoptotic factor in smooth muscle cells, a finding which would not have been predicted based on the anti-apoptotic role previously established for A20 in endothelial cells. The present invention thus provides therapeutic strategies for up-regulating pro-apoptotic factors in smooth muscle cells

25   for prevention and treatment of vascular disease.

Specifically, the present invention provides a method of treating vascular disease by (1) modifying mammalian smooth muscle cells so that

-2-

they exhibit increased anti-inflammatory and pro-apoptotic activity, and
(2) transplanting the smooth muscle cells into a transplant candidate or a
patient diagnosed with a vascular disease (e.g., atherosclerosis, transplant-
associated vasculopathy, or chronic rejection). Preferably, the smooth

5   muscle cells are selectively treated. For example, the present invention is
useful in the treatment and prevention of atherosclerosis and transplant-
associated vasculopathy (TAV). The increase in anti-inflammatory and
pro-apoptotic activity is accomplished by inserting into the smooth muscle
cell, or a progenitor thereof, DNA encoding A20 capable of decreasing

10   inflammation and increasing apoptosis of the smooth muscle cell.
Preferably the A20 protein employed by the methods of the invention also
achieves an anti-proliferative effect in the smooth muscle cell. A key
feature of the invention is that the methods include introduction of nucleic
acid to a tissue or organ having smooth muscle cells, such as a vessel, a

15   heart, a kidney or a liver into smooth muscle cells.

In a related aspect, the method of the present invention can be
applied to prevent TAV in a patient at risk for developing TAV. This
method involves modifying a mammalian smooth muscle cell by inserting
into the smooth muscle cell DNA encoding a protein capable of decreasing

20. inflammation and increasing apoptosis of the smooth muscle cell and
transplanting the smooth muscle cell (or tissue or organ having a smooth
muscle cell) into the patient. A patient classified as a patient at risk for
developing TAV would be, for example, a patient receiving an organ or
vessel transplant.

25   Where the method of the invention is applied to the prevention or
treatment of vascular disease, the anti-inflammatory/pro-apoptotic protein
is preferably the A20 protein. The present invention is based on the
discovery that the A20 protein, previously thought to be an anti-apoptotic

factor, has pro-apoptotic activity in smooth muscle cells. Included in certain aspects of the invention is the use of mutants and derivatives of the A20 protein that may have increased activity or expression in smooth muscle cells.

5   In another related aspect, the invention provides a method of treating atherosclerosis, or preventing TAV by transplanting donor smooth muscle cells, or graftable tissues or organs having smooth muscle cells into a mammalian recipient diagnosed with atherosclerosis or at risk for developing TAV. The first step requires modifying the donor smooth
10   muscle cells, or progenitor cells thereof, by inserting therein nucleic acid encoding the A20 protein. The second step requires transplanting the resultant modified donor smooth muscle cells, or tissue, or organ comprising the cells into the recipient and expressing in the cells the A20 protein, thereby substantially promoting apoptosis. Preferably, smooth
15   muscle cells modified by this procedure are capable of substantially inhibiting inflammation in the presence of a cellular activating stimulus (e.g., TNF).

The present invention also provides a method of preparing a vascularized organ or vessel for transplantation into a patient by obtaining
20   an organ or vessel for transplant and perfusing the organ with nucleic acid encoding the A20 protein. A variety of vectors are available in the art for gene transfer. Particularly preferred vectors are the adenovirus vector and the lentivirus vector. In a preferred embodiment, the organ or vessel is perfused with a gene transfer vector and an immunoregulatory factor (e.g.,
25   a cytokine). Alternatively, a therapeutic agent may be perfused into the organ or vessel to enhance the expression of A20.

Organ and vessel transfer procedures can be most effectively applied to patients that have suffered ischemia, reperfusion injury,

-4-

mechanical injury, immunologic injury, pharmacologic injury, or coronary trauma. Alternatively, methods of organ and vessel transfer can be applied to a patient who has undergone undergone balloon angioplasty. However, the most preferred application of the organ or vessel transfer procedure of

5   the present invention is in the treatment of patients diagnosed with atherosclerosis. Preferably, the organ or vessel being prepared is a vascularized organ transplant or a vascular graft.

The present invention also relates to a mammalian smooth muscle cell that is modified to express the A20 protein. Expression of the A20

10   protein imparts on the smooth muscle cell an anti-inflammatory/pro-apoptotic effect in the presence of a cellular activating stimulus. The modified smooth muscle cell thereby exhibits increased apoptosis, relative to an untreated smooth muscle cell. In preferred embodiments, the A20 protein also blocks smooth muscle cell proliferation. Similarly, the

15   present invention also relates to a donor smooth muscle cell, or a tissue or organ having a smooth muscle cell, for transplantation into a recipient species, wherein the cell is modified to express A20. Alternatively, a therapeutic agent may applied to the cell to enhance the expression of A20.

20   In another related aspect, the invention provides an organ or vessel from a non-human transgenic or somatic recombinant mammal comprising DNA encoding a pro-apoptotic protein of a different species. The non-human transgenic or recombinant mammal thus acts as a stable source of transplant tissue. Preferably, the non-human animal is porcine and the

25   pro-apoptotic factor is human.

The present invention also provides a method of identifying compounds that substantially promote apoptosis and substantially inhibit growth or proliferation in smooth muscle cells. The method requires

-5-

exposing smooth muscle cells to a test compound and assaying the smooth
muscle cells for increased apoptosis and reduced growth or proliferation.
A variety of methods are available for assaying increased apoptosis and
reduced growth or proliferation. Primarily, assays that measure A20

5 biological activity in smooth muscle cells (i.e., the ability to promote
apoptosis and inhibit proliferation) will be used to assess the effect of a
compound. Alternatively, these assays represent methods of detecting
binding of a compound to a pro-apoptotic protein of interest (i.e., A20).
Particularly preferred compounds include compounds that enhance A20

10 biological activity or increase the level of A20 in a smooth muscle cell.

     In another preferred embodiment, the present invention also
provides a method of treating a patient having a vascularized organ
transplant, a vessel transplant, or vascular disease by enhancing A20
biological activity. Therapeutic agents, such as the compounds identified

15 as described above, that enhance A20 biological activity, can be used to
treat patients having vascularized organ transplants, vessel transplants, or
vascular diseases. Such compounds can also be applied to the prevention
of vascular disease in a patient. Such compounds are preferably in the
form of a therapeutic agent, e.g., a cytokine, Fas, or a drug. Alternatively,

20 a mutant A20 gene might be employed that encodes an A20 protein that
exhibits increased A20 biological activity.

     The present invention also relates to a method of promoting
apoptosis in smooth muscle cells in a patient, by increasing the level of
A20 in the smooth muscle cells. One mechanism of achieving this goal is

25 to modify smooth muscle cells to express A20. Another mechanism by
which to achieve increased A20 expression in a smooth muscle cell is to
employ a mutant of the A20 gene encoding a protein that is expressed at a
higher level in A20 cells. Yet another mechanism by which the level of

A20 protein can be increased in a smooth muscle cell is by administration of a therapeutic agent to the smooth muscle cell, (e.g., a cytokine, a transcription factor, Fas, or a drug). Preferably, the method is used to treat a patient with a vascularized organ transplant, a vessel transplant or

5   vascular disease (e.g., TAV, or atherosclerosis).

Definitions

"Vascular disease," is used herein to refer to atherosclerosis, transplant-associated vasculopathy, chronic rejection, stenosis (e.g., vein graft stenosis or peri-anastomatic prosthetic graft stenosis), restenosis

10 (e.g., restenosis after angioplasty or stent placement, and the like), human atheroma, septic shock, and vasculitis. Vascular disease also refers to vascular conditions that develop after a surgical treatments, such as, venous bypass surgery, balloon angioplasty, post-angioplasty of atherosclerotic plaques of both coronary and peripheral arteries, and allo-

15 and xenograft rejection. Alternatively, vascular disease is used to refer to the disease of a patient that has suffered ischemia, reperfusion injury, mechanical injury, immunologic injury, pharmacologic injury of a vessel, or coronary trauma. In yet another embodiment, the patient has undergone balloon angioplasty.

20   "A20" refers to a natural mammalian A20 gene, including the cDNA or protein thereof. A20 also include derivatives having variations in the cDNA (SEQ ID NO:1) or amino acid (SEQ ID NO:2) sequence. Included in the definition of A20 are derivatives of the A20 protein having conserved amino acid substitutions. In addition, A20 includes mutants

25 having alterations in one or more amino acids that either maintain or increase the activity of A20. The A20 gene or protein employed in the invention may, for example, be porcine, bovine, or primate (e.g., a

human), depending on the nature of the cells to be modified and the intended recipient species for transplantation.

By "A20 gene" is meant a gene encoding a polypeptide having A20 pro-apoptotic/ anti-inflammatory activity in smooth muscle cells or other

5   biological activities described herein. An A20 gene is a gene encoding an A20 polypeptide having about 60% or greater, more preferably 70% or greater amino acid sequence identity to the A20 amino acid sequence of SEQ ID NO:2, or a portion thereof. For example, the gene may encode human or murine A20 polypeptide. An A20 gene may also be defined as

10 encoding a polypeptide with at least 50% of the activity of the A20 polypeptides described below. Preferably, the A20 gene is a human or murine A20 gene. In a further aspect, the A20 gene of the invention is at least 70%, and more preferably at least 80%, or at least 90% homologous to SEQ ID NO:1.

15 A protein or polypeptide "having A20 activity" or "A20 active protein" refers to a protein which is able to block or suppress nuclear factor κB (NF-κB) activation, and which is at least 70%, preferably at least 80%, and more preferably at least 90% (most preferably at least 95%) homologous to the protein sequence of a natural mammalian A20 protein

20 (e.g., human) (SEQ ID NO:2) (see, Dixit et al., *J. Biol. Chem.*, (1990) 265:2973, and WO 05/0062, which are hereby incorporated by reference). Preferably, by blocking is meant at least 50% inhibition of IκB degradation that occurs after TNF stimulation, but blocking can also mean a 50% or greater inhibition of NFκB binding to κB elements on the DNA,

25 or a 50% or greater decrease in the up-regulation of mRNA seen for NFκB-regulated genes following TNF treatment, such genes include, for example, ICAM-1 and MCP-1.

By "therapeutic gene" is meant a DNA that achieves an anti-proliferative, pro-apoptotic effect in smooth muscle cells. In addition, the therapeutic gene may also have the effect of decreasing inflammation, (e.g., by inhibiting NF-κB activation in a smooth muscle cell).

5   "Inflammation" refers to a cellular response to TNF that constitutes in part activation of NF-κB. An "anti-inflammatory" is a permeable factor (protein, peptide, or chemical agent (i.e., a drug)) that suppresses, inhibits or blocks the cellular response to TNF and NF-κB activation.

      "Host" or "recipient" refers to the body of the patient into whom

10   donor biological material is grafted. Preferably, the host is an animal which is of interest for experimental investigations, providing a model for treatment of human disease. More preferably, the host is a mammal or patient, and may be any mammalian species. For example, the mammalian host may be a rodent (e.g., including mice, rats and hamsters),

15   or another mammal (e.g., rabbits, equines, bovines, canines, felines, etc.). Such mammals are of interest for the study of experimentally induced vascular lesions. A particularly preferred host is a primate. A most preferred mammalian host is a human.

      A "patient at risk" for developing TAV may include all patients

20   receiving allo- or xenogeneic tissue or organ transplants.

      A "vascularized organ transplant," according to the present invention, includes any organ having vasculature and containing smooth muscle cells. For example, the heart, liver, and kidney are vascularized organs that can be used for transplant according to the present invention.

25   "Vascularized organ transplant," as used herein, refers to any organ that may be used in a transplant procedure to replace a like organ that has suffered end-stage organ failure.

"Graft," "transplant", or "implant" are used interchangeably to refer to biological material derived from a donor for transplantation into a recipient, and to the act of placing such biological material in the recipient.

"Allogeneic" refers to the donor and recipient being of the same

5   species (also, allograft). As a subset thereof, "syngeneic" refers to the condition wherein donor and recipient are genetically identical. "Autologous" refers to donor and recipient being the same individual. "Xenogeneic" (and "xenograft") refer to the condition where the graft donor and recipient are of different species.

10   By "constitutive" when used in reference to a promoter is meant substantially continuous transcription of the gene and expression of the protein over the life of the cell.

By "regulable" when used in reference to a promoter is meant that the transcription of the gene and expression of the protein is related to the

15   presence, or absence, of a given substance. An embodiment of "regulable" expression includes "inducible" expression, i.e. whereby transcription (and thus protein expression) occurs on demand in response to a stimulus. The stimulus may comprise activating stimuli or a predetermined external stimulus. The activating stimuli may be any of the stimuli which give rise

20   to changes in the endothelium or smooth muscle cells of donor tissue or organs which contribute to vascular disease. The predetermined external stimulus may be the presence of a drug, cytokine, or other agent. An example of a drug stimulus is tetracycline, which has the ability to activate the tetracycline responsive promoter. Alternatively, the activating stimuli

25   may be TNF mediated NF-κB activation.

"Candidate compound," "test compound," "agent," or "therapeutic agent" as used herein include any molecule or permeable factor, e.g., a protein or pharmaceutical, i.e., a drug, with the capability of substantially

-10-

promoting apoptosis and substantially inhibiting smooth muscle cell
growth or proliferation.

By "A20 biological activity" as used herein, is meant, the ability of
A20 to decrease inflammation and increase apoptosis in smooth muscle
5 cells. Preferably, A20 biological activity also includes the ability to
decrease proliferation of smooth muscle cells. A variety of assays are
described herein (see, Examples) that measure A20 biological activity.

By "enhanced biological activity" as used herein in reference to the
A20 protein, is meant, that the pro-apoptotic/anti-proliferative activity of
10 the A20 protein has been increased above that of the wild-type A20
protein.

By "increased level" as used herein, is meant that the A20 protein is
present in a greater quantity or has greater biological activity in a modified
smooth muscle cell compared to the level of A20 protein in an unmodified
15 wild-type smooth muscle cell.

Brief Description of the Drawing

Fig. 1 is a photograph of a Western blot depicting A20 expression
induction by TNF (panel A) and a photograph of immunohistochemical
analysis of A20 expression in smooth muscle cells after TNF treatment
20 (panel B).

Fig. 2 is a photograph of an electrophoretic mobility shift assay
(EMSA) depicting inhibition of NF-κB binding to DNA upon A20
expression (panel A) and a photograph of a Western blot depicting
inhibition of IκBα degradation in the presence of A20 (panel B).

25 Fig. 3 is a photograph of a Northern blot depicting inhibition of
ICAM-1 up-regulation by A20.

Fig. 4 is a graph depicting inhibition of smooth muscle cell proliferation by A20.

Fig. 5 is a graph depicting A20-induced apoptosis of smooth muscle cells in response to cytokine treatment.

5   Fig. 6 is a photograph of immunohistochemical analysis of A20 expression in heart vessels of control and transgenic mice.

Fig. 7 is a photograph of a Northern blot showing that overexpression of A20 in SMC inhibits TNF-mediated up-regulation of MCP-1 mRNA as compared to not infected (NI) and rAd.β-gal-infected

10   cells.

Fig. 8 depicts the percent of not infected (NI), rAd.A20 infected, or rAd.β-gal infected cells undergoing apoptosis in response to various treatments.

Fig. 9 is a photograph of a Northern blot showing that

15   overexpression of A20 in SMC inhibits αFas-mediated up-regulation of MCP-1 mRNA and also down-regulates the constitutive level detected in not infected (NI) cells.

Fig. 10 is a photograph of a Western blot indicating that expression of A20 in SMC does not modify constitutive levels of Bcl-2 or Bcl-x$_L$

20   expression, nor their levels upon addition of cytokines.

Description of the Preferred Embodiments

The present invention provides an alternative approach to inhibiting atherosclerosis through modifying cells in the vessel wall, particularly

25   smooth muscle cells, that appear to be involved in the development of lesions associated with vascular disease, particularly atherosclerosis and TAV. This approach may offer the advantage of protecting against atherosclerosis and TAV regardless of the factors responsible for their

initiation. According to the present invention, a "therapeutic gene"
encoding and expressing a therapeutic protein achieves an
anti-inflammatory, pro-apoptotic effect in smooth muscle cells (SMC).
Preferably, the therapeutic gene also achieves an anti-proliferative effect in

5   smooth muscle cells. Most preferably, the therapeutic gene also achieves
an anti-inflammatory effect in endothelial cells (EC).

Atherosclerosis and Transplant Associated Vasculopathy

Atherosclerosis may be viewed as a chronic inflammatory response
to high concentrations of oxidized LDL (Steinberg, *Nutrition and*

·10 *Biotechnology in Heart Disease and Cancer*, 1995 pages 39-48; Ross, *N.*
Engl. J. Med., (1986) 314:488-500). This response consists of a series of
cellular and biochemical events. The present invention is based on the
concept that mediators released by cells in the lesion may contribute to the
development of atherosclerosis.

15   Although the initiating factors of atherosclerosis and TAV are
different, fundamental pathological features are similar and relate to the
dysregulation of the two major cell types of the vessel wall: SMC and EC.
SMC contribute to the atherosclerotic lesion by essentially three
mechanisms: (i) promotion of inflammation, (ii) aberrant SMC

20   proliferation and migration within the neointima, and (iii) dysregulated
SMC apoptosis (Ross, *supra*, Pomerantz et al., *Nutrition and*
Biotechnology in Heart Disease and Cancer 1995 page 4964; Rekhter et
al., *Am. J. Pathol.* (1995) 147:668-667; Bennett, *Cardiovasc. Res.* (1999)
41:361-368; Pollman et al., *Nat. Med.* (1998) 4:222-227; Rekhter, *Cardio.*

25 *Res.* (1999) 141:376-384; Koyama et al., *Cell.* (1996) 87:1069-1078). EC
cells contribute to the development of the atherosclerotic lesion by
acquiring an inflammatory phenotype that promotes leukocyte infiltration,

-13-

macrophage infiltration, coagulation and platelet aggregation. EC cell
activation further leads to thrombosis and ischemia, foam cell
development, matrix deposition, as well as promotion of SMC migration
and proliferation in the neointima (Cotran et al., *J. Am. Soc. Nephrol.*

5   (1990) 1:225-235; Gimbrone, *American Journal of Cardiology* (1995)
75:67B-70B).

Transplant-associated vasculopathy (TAV) is the main feature of
chronic allograft rejection. TAV is a form of accelerated atherosclerosis
resulting from a chronic inflammatory process initiated by the immune

10 response to the allograft (Tullius et al., *Transplantation* (1995) 59:2126-
2128; Ardehali et al., *Circulation* (1995) 92:450-456). Pathologically,
with the exception of foam cell development, the lesions of TAV are quite
similar to those of the atherosclerotic plaque. The present invention
provides insight into the pathogenesis of these related diseases and the

·15 regulatory processes which maintain the structure and homeostatic
functions of a healthy vessel. Through this understanding, a strategy for
protecting vessels from atherosclerosis and TAV is proposed.

A20

A20 is a zinc finger protein originally identified as a tumor necrosis

20 factor (TNF)-inducible gene in human umbilical vein endothelial cells
(HUVEC) (Opipari et al., *J. Biol. Chem.* (1990) 265:14705-14708). A20
is expressed in a variety of cell types in response to a number of stimuli
such as interleukin (IL)-1 in HUVEC, CD40 cross-linking in B cells,
phorbol 12-myristate 13-acetate (PMA) or HTLVI Tax protein in Jurkat T

25 cells, Epstein Barr Virus latent membrane protein-1 and
lipopolysaccharide (LPS) (Tewari et al., *J. Immunol.* (1995) 154:1699-

1706; Laherty et al., *J. Biol. Chem.* (1992) 267:24157-24160; Sarma et al.,
J. Biol. Chem. (1995) 270:12343-12346). It has been established that
expression of A20 in various cell types, including EC cells, confers
resistance to TNF-mediated apoptosis (Tewari et al., *supra*; Opipari et al.,

5   *J. Biol. Chem.* (1992) 267:12424-12427; Ferran et al., *Blood* (1998)
91:2249-2258). Thus, A20 has been thought of as an anti-apoptotic factor.

Morphologically, apoptosis is characterized by loss of contact with
neighboring cells, concentration of cytoplasm, endonuclease activity-
associated chromatin condensation, pyknosis, and segmentation of the

10   nucleus. Disappearance of microvilli from the cell surface and vesicle
formation on the cell surface (membrane blebbing) are also observed. The
remaining fragments of apoptotic body cells are phagocytosed by
neighboring cells (see, Duvall, E. and Wyllie, A.H., *Immunology Today*
(1986) 7(4):115-119).

15   Traditionally, apoptotic cell death is considered to be of
fundamental importance in inflammation, embryogenesis, and lymphocyte
selection. Avoidance of cell activation and apoptotic cell death
accompanying inflammation, particularly in connection with organ
transplantation, has become a major goal for researchers in the art. In

20   endothelial cells, graft injury and loss occurring in connection with graft
preservation techniques and accompanying graft rejection exemplify the
need for prevention of such a process. However, the present invention is
based on the premise that promotion of apoptosis may actually be
desirable for graft preservation and prevention of atherosclerosis and TAV

25   in certain cell types, including smooth muscle cells.

The cDNA and deduced amino acid sequences of the human A20
gene obtained from HUVEC were published by Opipari et al. (*J. Biol.
Chem.* (1992) 267:12424-12427), as indicated herein above. At the

protein level, the deduced sequence of 790 amino acids contains within its
carboxyl terminal half 7 Cys_2/Cys_2 zinc finger repeats; six with the
configuration $Cys-X_4-Cys-X_{11}-Cys-X_2-Cys$ and one with the configuration
$Cys-X_2-Cys-X_{11}-Cys-X_2-Cys$, wherein X is any amino acid and the

5   subscripts represent numbers of amino acids between each of the indicated
cysteines. The zinc finger repeat region is important for activity of A20 in
endothelial and other cell types. A novel finger loop domain composed of
11 amino acid residues has also been identified (see Opipari et al., supra).
In one embodiment of this invention, the protein having A20 activity

10   comprises amino acid residues 386-790 of SEQ ID NO:2, comprising the
zinc finger region of the native protein sequence, or a region at least 80%
homologous to the residues. Another suitable truncated form of the native
human protein consists essentially of residues 373-790 of SEQ ID NO:2.
A person skilled in the art will appreciate that mutants useful to the

15   invention may include the zinc finger region of the protein but are not
exclusive to the zinc finger region of the protein (see, Natoli et al. J. Biol.
Chem. (1998) 273:31262-31272). The mutants may be identified by
introducing specific desired mutations into a particular region of the A20
gene and testing the resultant mutant protein for a desired activity.

20   In vitro, A20 has a dual "cytoprotective" role in EC because it is
both anti-apoptotic and anti-inflammatory. In vivo, A20 is expressed in
EC and SMC of long term surviving hamster to rat heart xenografts and is
associated with the absence of inflammation, apoptosis, and of TAV in
long term surviving hamster to rat xenograft (Bach et al., Nature Medicine

25   (1997) 3:196-204). It has been demonstrated that the anti-inflammatory
effect of A20 is mediated via blockade of the transcription factor NF-κB
(see Grilli et al., International Review of Cytology, (1993) 13:1-61).

A20 Function In SMC and EC

NF-κB is a transcriptional activator associated with immediate early gene expression in EC activation. NF-κB is usually sequestered in the cytoplasm in association with its inhibitor IκBα (Baldwin, *Ann. Rev.*

5 *Immunol.* (1996) 14:649-681). Upon exposure to stimuli such as lipopolysaccharide (LPS), TNF, or oxygen radicals, IκBα is phosphorylated, ubiquitinated and subsequently degraded in the proteasome allowing translocation of NF-κB to the nucleus of the EC (Traenckner et al., *EMBO J.* (1994) 13:5433-5441; Traenckner et al., *J.*

10 *Cell Sci.* Suppl. (1995) 19:79-84). In the nucleus, the binding of NF-κB to certain NF-κB binding sites (kB elements) in promoter regions of the nuclear DNA initiates transcription of genes directly or indirectly under the control of these promoters. This leads to *de novo* expression in EC of a number of NF-κB-dependent genes including adhesion molecules,

15 chemokines, and pro-thrombotic factors that are the hallmarks of the inflammatory phenotype (Read et al., *J. Exp. Med.* (1994) 179:503-512; Bach et al., *Immunological Reviews*, (1994) 141:5-30; Collins, *Lab. Invest.* (1993) 68:499-508). EC activation promotes inflammation and can lead to apoptosis of these cells.

20 Prior to the present invention, evidence from EC suggested that A20 belongs to a sub-set of TNF-inducible genes which assist in ultimately conferring resistance to TNF-induced apoptosis in EC (see, Tewari et al., *J. Immunol.* (1995) 1699-1706; Ferran et al., *Blood* (1998) 91:2249-2258; Opipari et al., *J. Biol. Chem.* (1990) 285: 14705-14708;

25 Dixit et al., *J. Biol. Chem.* (1990) 264, 16905-16909). It was shown that TNF induces expression of A20 in EC, through NF-κB binding sites located in the A20 promoter, extending from -45 to -54 (5'-GGAAATCCCC-3') (SEQ ID NO:3) and from -57 to -66 (5'-

GGAAAGTCCC-3') (SEQ ID NO:4) of the A20 gene (Laherty et al., *J. Biol. Chem.* (1993,)268, 5032-5039; Krikos et. al., *J. Biol. Chem.* (1992) 267:17971-17976).

The proliferation of SMC, like EC, requires activation of the

5   transcription factor NF-κB, as demonstrated *in vitro* and in atherosclerotic plaques *in vivo* (Brand et al., *J. Clin. Invest.* (1996) 97:1715-1722; Bourcier et al., *J. Biol. Chem.* (1997) 272:15817-15824; Bellas et al., *J. Clin. Invest.* (1995) 96:2521-2527). Activation of SMC provides a critical patho-physiological basis for a variety of conditions, including allo- and

10 xenograft rejection, vasculitis, as well as atherosclerosis and TAV. Inflammatory cytokines, CD40-CD40L cognate interactions, oxidized lipids, oxidative stress, as well as growth factors present in both human atheroma and chronic transplant vasculopathy can activate NF-κB and elicit specific functions in SMC (Obata et al., *Biochem. Bioph. Res. Com.*

15 (1996) 224:27-32; Mach et al., *Proc. Natl. Acad. Sci USA* (1997) 94:1931-1936).

The mode of activation of SMC proliferation via NF-κB remains unclear. However, without limiting the biochemical mechanism of the invention, we propose that NF-κB may drive the cell cycle through

20 induction of cyclin D1, in the mid-G1 phase of the cell cycle (Hinz et al., *Mol. Cell. Biol.* (1999) 19:2690-2698; Sherr et al., *Cell.* (1993) 73:1059-1065; Roberts, *Cell.* (1999) 98:129-132). Inhibition of the cell cycle in SMC inhibits SMC proliferation and has been demonstrated to redirect the vascular disease response away from neointimal hyperplasia and

25 toward medial hypertrophy (Mann et al., *Proc. Natl. Acad. Sci. USA* (1995) 92:4502-4506; Chang et al., *Science* (1995) 267:518-522; Kato et al., *Exp. Mol. Pathol.* (1999) 66:39-52; Yang et al., *Proc. Natl. Acad. Sci. USA* (1996) 93:7905-7910). In addition to its anti-proliferative effect,

blockade of NF-κB would also serve an anti-inflammatory function by inhibiting the up-regulation of NF-κB dependent genes that are deleterious for the development of atherosclerosis (e.g., adhesion molecules ICAM-1 and VCAM-1, pro-inflammatory receptors CD40 and CD40L, growth

5   factors, and matrix proteinases) (Ross et a., *supra*; Ardehali et al., *supra*; Mach et al., *Nature* (1998) 394:200-203).

Clinical trials aimed at blocking a single growth or inflammatory factor have failed to show efficacy. We concluded that this is not surprising given the redundancy of the system via the multiplicity of

10   growth factors and pro-inflammatory mediators that contribute to the pathogenesis of atherosclerosis. We proposed that a more successful approach may be to target the components of intracellular signaling cascades that are shared in response to many of these growth regulatory molecules and pro-inflammatory stimuli. We reasoned that if A20 inhibits

15   NF-κB activation in SMC, as it does in EC, A20 expression could be instrumental in inhibiting SMC proliferation and protecting vascular tissues and organs from atherosclerosis and TAV.

Thus, A20 provides a critical pathophysiological basis of a variety of conditions, including allo- and xenograft rejection, vasculitis and

20   atherosclerosis. U.S.S.N. 08/601,515, filed February 14, 1996, incorporated herein by reference, discloses the use of gene therapy techniques utilizing A20 and other anti-apoptotic/anti-inflammatory genes and their expression products to prevent and treat inflammation in endothelial cells.

25   We further hypothesized that if A20 inhibits NF-κB activation in SMC as it does in EC, A20 expression could block SMC proliferation and prevent or reduce atherosclerosis and TAV. Without limiting the biochemical mechanism of the invention, we proposed that the underlying

-19-

mechanism of atherosclerosis and TAV is NF-κB stimulation of SMC proliferation. Surprisingly, upon testing this hypothesis, we found that A20, typically thought to act as an anti-apoptotic factor, acts as a pro-apoptotic factor in SMC.

5   *Therapeutic Strategies*

        The present invention provides therapeutic strategies directed at up-regulating pro-apoptotic factors (or down-regulating anti-apoptotic factors) for inhibition of vascular lesion formation and prevention or treatment of vascular disease. Preferably, the protective gene should achieve an anti-

10   proliferative and pro-apoptotic effect in SMC. According to the present invention, protection of SMCs in the vessel wall that appear to underlie the development of lesions associated with atherosclerosis, TAV, and other vascular diseases can be achieved by modifying the SMC so that it expresses a pro-apoptotic factor.

15   We demonstrate herein that A20 is expressed in SMC in response to TNF (a pro-inflammatory stimulus), establishing that A20 expression as part of the physiological response of SMC to injury (see, Example 1). In addition, we show that overexpression of A20 in SMC inhibits NF-κB activation in response to TNF (see, Example 1). This NF-κB blockade in

20   SMC correlates with suppression of NF-κB-dependent pro-inflammatory gene expression (e.g., ICAM-1) and SMC proliferation, both of which are involved in the development of atherosclerosis.

        As noted above, we also discovered that expression of A20, to date viewed as an anti-apoptotic gene, sensitizes SMC to cytokine-mediated

25   apoptosis, a surprising finding. This novel function of A20 in SMC further augments its anti-atherogenic potential by combining anti-proliferative and pro-apoptotic functions. Interestingly, SMC seem

very resistant to apoptotic stimuli *in vitro*, including nitric oxide, oxidized

LDL, high levels of cytokines (IFN-γ, TNF-α an IL-1β), anti-oxidants

such as PDTC, Fas-mediated apoptosis, and growth factor deprivation

(Geng, et al., *Arterioscler. Thromb. Vasc. Biol.* (1996) 16:19-27; Tsai et

5   al., *J. Biol. Chem.* (1996) 271:3667-3670; Björkerud et al., *Arterioscler.*

Thromb. Vasc. Biol. (1996) 16:415-424; Pollman et al., *Circ. Res.* (1996)

79:748-756; Fox et al., *J. Biol. Chem.* (1996) 271:12578-12584).

Furthermore, we found that sensitization of SMC to apoptosis by

expressing A20 is more potent than for cells expressing IκBα (the specific

10 inhibitor of NF-κB), suggesting that A20 has targets in SMC other than

NF-κB.

The present invention establishes that modification of SMC to

express A20 may protect vessels and vascularized organs from

atherosclerosis and TAV. The results described herein combined with our

15 finding that A20 is expressed in SMC of long term surviving hamster to

rat heart xenografts lacking transplant arteriosclerosis (Bach et al., *supra*)

lend support to this proposal. Thus, the present invention sets the basis for

the use of A20 and other pro-apoptotic factors as gene therapy tools in

pre-clinical and clinical protocols. This approach has immediate clinical

20 potential in vascular conditions.

The present invention provides a method of treating vascular

disease by (1) modifying a smooth muscle cell by inserting into the

smooth muscle cell a therapeutic gene that achieves an anti-inflammatory,

pro-apoptotic, effect in smooth muscle cells, and (2) transplanting the

25 smooth muscle cell, or tissue, or organ comprising the smooth muscle cell

into a patient diagnosed with a vascular disease or a transplant candidate

(i.e., a patient having end-stage organ failure).

Alternatively, the method of the invention can be used to prevent vascular disease, for example TAV, by (1) modifying a smooth muscle cell by inserting into the smooth muscle cell a therapeutic gene that achieves an anti-inflammatory, pro-apoptotic effect in smooth muscles

5   cells, and (2) transplanting the smooth muscle cell, or tissue, or organ comprising the smooth muscle cell into a patient at risk for developing TAV. Patients at risk for developing TAV include patients receiving allo- or xenogeneic tissue or organ transplants. For example, a patient scheduled to receive a heart transplant would be classified as a patient at

10 risk for developing TAV.

According to the present invention, a therapeutic gene is a DNA sequence encoding a protein capable of decreasing inflammation (e.g., by inhibiting NF-κB activation in a smooth muscle cell) and increasing apoptosis in a smooth muscle cell, thus acting as a dual/anti-

15 inflammatory, pro-apoptotic factor. In preferred embodiments, the therapeutic gene also achieves an anti-proliferative effect in smooth muscle cells. Most preferably, the therapeutic gene further achieves an anti-inflammatory effect in endothelial cells in addition to smooth muscle cells. The therapeutic gene A20 has been demonstrated to have anti-

20 inflammatory, anti-proliferative, and pro-apoptotic activities in smooth muscle cells and also retains an anti-inflammatory effect in EC. Thus, according to a most preferred embodiment of the invention, A20 is the therapeutic gene used in the present invention. Alternatively, the present invention utilizes a therapeutic gene encoding a mutant or derivative of the

25 A20 protein.

In a related aspect, the invention comprises a method of treating a dysfunctional activation response of a smooth muscle cell to an inflammatory or other activation stimulus, by inserting into the smooth

muscle cell, DNA encoding a polypeptide having the pro-apoptotic
activity of an A20 protein in operative association with a suitable promoter
and expressing the polypeptide at effective levels, whereby apoptosis in
the smooth muscle cell is substantially activated. The DNA sequence

5   including the pro-apoptotic protein or polypeptide may also include, for
example, a transcriptional termination region and an appropriate signal
sequence, e.g., a mitochondrial specific signal sequence to provide site-
specific targeting ability to the pro-apoptotic factor.

The promoter and/or the protein encoding region of the present

10   invention may be heterologous (i.e., non-native) to the cell. Alternatively,
one or both of the protein encoding region and the promoter region may be
native to the cell. The promoter may be a promoter other than the
promoter which normally controls expression of the pro-apoptotic factor
(e.g., A20) in the cell. Preferably the promoter controlling the expression

15   of the protein encoding region is a constitutive, regulable, and/or tissue
specific promoter. One feature of a regulable promoter is that it may be
inducible, that is, transcription (and thus protein expression) can be
activated on demand in response to a stimulus. An advantage of
employing an inducible promoter for transplantation purposes is that the

20   desired high level expression of the pro-apoptotic protein or polypeptide
(e.g., A20) can be obtained on demand in response to a predetermined
stimulus, such as e.g., the presence of tetracycline in the cellular
environment. An example of a tetracycline-inducible promoter which is
suitable for use in the invention is disclosed by Furth et al., (*PNAS USA*

25   (1994) 91:9302-9306). A regulable promoter system in which
transcription is initiated by the withdrawal of tetracycline is described by
Gossen and Bujard, (*PNAS USA* (1992) 90:5547-51).

Preferably, expression of the anti-inflammatory/pro-apoptotic
protein or polypeptide is induced in response to a predetermined external
stimulus, and the stimulus is applied immediately prior to subjecting the
SMC to an activating stimulus, so that expression, for example, of A20, is

5   already at effective levels to block NF-κB activation and thereby inhibit
inflammation and promote apoptosis. Preferably, the anti-
inflammatory/pro-apoptotic protein or polypeptide is also expressed at a
level sufficient to block smooth muscle cell proliferation. The activities of.
blocking inflammation and proliferation are clearly anti-atherogenic.

10   It is preferred that the invention also includes the smooth muscle
cells, modified as described above, and tissues or organs comprising
smooth muscle cells. Accordingly, the invention provides a smooth
muscle cell modified to express A20, or a mutant or derivative of A20. In
preferred embodiments, the A20 protein is capable of inhibiting

15   inflammation and promoting apoptosis in the presence of a cellular
activating stimulus. As a result, the modified smooth muscle cell exhibits
increased apoptosis relative to an untreated smooth muscle cell.
Preferably, the A20 protein is also capable of blocking smooth muscle cell
proliferation. Accordingly, the present invention further provides a donor

20   smooth muscle cell, or a tissue, or organ having the donor smooth muscle
cell, for transplantation into a recipient species, wherein the donor cell is
modified to express A20.

In yet another related aspect, the present invention provides an
organ or vessel from a non-human transgenic or somatic recombinant

25   mammal containing DNA encoding a pro-apoptotic protein of a different
species. Preferably, the non-human organ or vessel is a porcine organ or
vessel that has been modified to express a pro-apoptotic factor. The pro-
apoptotic factor is preferably human, for example human A20.

-24-

Such cells, tissues and organs as described above can be used in surgical procedures such as heart, liver, or kidney transplantation. Additionally, a modified vessel according to the present invention, can be used, for example, as a vascularized organ transplant and in vascular

5   surgery.

In a related aspect, the present invention provides a method of preparing a vascularized organ or vessel for transplantation into a patient including (1) obtaining an organ or vessel for transplant; and (2) perfusing the organ with nucleic acid encoding A20 protein, or mutants or

10   derivatives thereof. Preferably, the prepared organ or vessel is a vascularized organ or a vascular graft. The prepared vascularized organ or vessel can then be used, for example, in a transplantation procedure to treat a patient that has suffered ischemia, reperfusion injury, mechanical injury, immunologic injury, pharmacologic injury, coronary trauma or

15   other vascular disease. Alternatively, the modified vascularized organ or vessel can be used in a patient who has undergone balloon angioplasty. Most preferably, the modified vascularized organ or vessel is used to replace a diseased vascularized organ or vessel.

For example, cells of a donor mammal (e.g., porcine) may be

20   modified according to the invention by insertion of a pro-apoptotic gene (e.g., porcine or human) under the control of a promoter which is inducible by a drug such as, for example, tetracycline. The animal, whether a somatic recombinant or a transgenic, may be raised to the desired level of maturity under tetracycline-free conditions, until such time as the cells, or

25   tissue, or organs comprising the cells, are to be surgically removed for transplantation purposes. In such case, prior to surgical removal of the organ, the donor animal may be administered tetracycline in order to begin inducing high levels of expression of the anti-apoptotic protein. The organ

-25-

can then be transplanted into a recipient (e.g., human), and tetracycline may continue to be administered to the recipient for a sufficient time to maintain the protein at the desired levels in the transplanted cells to inhibit NF-κB activation.

5   Alternatively, after being surgically removed from the donor, the organ can be maintained *ex vivo* in a tetracycline-containing medium until such time as grafting into a recipient is appropriate.

Preferably, the inserted DNA sequences are incorporated into the genome of the cell. Alternatively, the inserted DNA sequences may be

10   maintained in the cell extrachromosomally, either stably or for a limited period.

The modification of smooth muscle cells or endothelial cells, according to the invention, may be carried out *in vivo* or *ex vivo*. Thus, in one aspect, the invention provides a method for substantially promoting

15   apoptosis and substantially inhibiting inflammation in smooth muscle cells by applying an activation stimulus *in vivo*, to a patient in need of such therapy. The method includes modifying smooth muscle cells of a patient *in vivo* by inserting into the smooth muscle cells, DNA encoding a pro-apoptotic protein in operative association with a constitutive or inducible

20   promoter and expressing the protein at effective levels, whereby NF-κB activation is also substantially inhibited.

Nucleic acid molecules are provided in solution or in any other pharmacologically suitable form for administration. Conveniently, the subject compositions are administered intravascularly, particularly

25   intraarterially. The target neointimal lesion may be subject to isolation, so as to limit the flow of blood or allow for an extended period of incubation. For example, the blood vessels of an organ (e.g., a kidney) can be temporarily clamped off from the blood circulation of the patient, and the

vessels perfused with a solution comprising a transmissible vector
construct containing the subject pro-apoptotic gene, for a time sufficient
for at least some cells of the organ to be modified by insertion therein of
the vector construct. Upon removal of the clamps, blood flow can then be

5   restored to the organ and its normal functioning resumed. Alternatively,
the active agents may be maintained in the region by virtue of having a
specific affinity for target cells (perhaps via a targeting molecule), or may
be allowed to flow through the vasculature. The time of incubation
sufficient to deliver the nucleic acids to the cells of the vasculature is

10   usually less than about one hour, more usually less than about 30 minutes,
but may be for any period of time that is both effective and practical for
the *in vivo* isolated segment of blood vessel.

There are many delivery methods known in the art for enhancing
the uptake of nucleic acids by cells. Useful delivery systems include the

15   Sendai virus-liposome delivery systems (see Rapaport and Shai (1994) *J.
Biol. Chem.* 269:15124-15131), cationic liposomes, polymeric delivery
gels or matrices, porous balloon catheters (Shi et al. (1994) *Circulation*
90:955-951; and Shi et al. (1994) *Gene Therapy* 1:408-414), intraluminal
pressure (PCT/US96/06271, herein incorporated by reference), retrovirus

20   expression vectors, and the like.

The use of liposomes as delivery vehicles is one particular method
of interest. The liposomes fuse with the cells of the target site and deliver
their contents intracellularly. As noted above, liposomes are maintained in
contact with the cells for a time sufficient for fusion, using various means

25   to maintain contact, such as isolation, binding agents, and the like.
Liposomes may be prepared with purified proteins or peptides that mediate
fusion of membranes, such as those of the Sendai virus or influenza virus,
etc. The lipids may be any useful combination of known liposome

forming lipids, including, for example, cationic lipids, such as phosphatidylcholine. The remaining lipid will normally be neutral lipids, such as cholesterol, phosphatidyl serine, phosphatidyl glycerol, and the like.

5   Liposomes may be prepared by the procedure described by Kato et al. (*J. Biol. Chem.* (1991) 266:3361). This method allows for the incorporation into the lumen, high molecular weight molecules, particularly nucleic acids of one kilobase pair or more. In this way oligonucleotides, plasmids, large genes, chromosomal fragments, viruses

10 or viral segments may be introduced into cells efficiently.

       In another aspect, cell populations can be removed from the patient or donor animal, modified *ex vivo* by insertion of vector DNA, and re-implanted into the patient or transplanted into another recipient. For example, an organ can be removed from a patient or donor and subjected

15 to the perfusion step described above *ex vivo*. Following perfusion with the gene construct, the organ can be re-grafted into the patient or implanted into a different recipient of the same or different species.

       Gene transfer could also be performed in allografts or xenografts using *ex vivo* transduction of the organ prior to transplantation. For

20 xenotransplantation, transgenesis could be planned and is a well known technique in the field (see below). If successful, such an approach could be applied to all vascularized organs, especially the heart where allograft vasculopathy has emerged as the leading cause of death among transplant recipients after the first year.

25   For gene delivery, a variety of vectors or plasmids are available (see, Maniatis et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y.; Ausubel et al., *Current Protocols in Molecular Biology,* Greene Publishing Associates, New York, V. 1&2 1996; Harlow

and Lane Antibodies, A Laboratory Manual, Cold Spring Harbor
Laboratory, 1988, incorporated by reference herein). The common vectors
described herein are able to transiently or stably be maintained in smooth
muscle cells, usually for a period of at least about one day, more usually

5 for a period of at least about several days.

Retroviral vectors (particularly lentivirus vectors, which can infect
non-replicating cells), and replication-defective retroviral vectors lacking
one or more of the gag, pol, and env sequences required for retroviral
replication, are well-known to the art and may be used to transform

10 endothelial or other mammalian cells. PA 317 cells or other producer cell
lines producing helper-free viral vectors are well-described in the
literature. A representative retroviral construct comprises at least one viral
long terminal repeat and promoter sequence upstream of the nucleotide
sequence of the therapeutic substance and at least one viral long terminal

15 repeat and polyadenylation signal downstream of the sequence.

Vectors derived from adenoviruses, i.e. viruses that cause upper
respiratory disease in humans and are present in latent infections in
primates, are also generally known in the art and are particularly preferred
for use in the present invention. The ability of adenoviruses to attach to

20 cells at low ambient temperatures is an advantage in the transplant setting
which can facilitate gene transfer during cold preservation of tissues or
organs. Adenoviral mediated gene transfer into vessels or organs by
means of transduction perfusion is also a means of modifying cells *in vivo*.
(Guzman et al. *Circulation* (1993) 88:2838-2848; Lemarchand,

25 *Circulation Res.* (1993) 72:1132-1138)

Accordingly, the method of the present invention further provides a
method of preventing or treating vascular disease, for example,
atherosclerosis and TAV, by transplanting donor smooth muscle cells, or

graftable tissues or organs that are composed of smooth muscle cells, to a mammalian recipient having vascular disease or end-stage organ disease (i.e., any transplant candidate) by (1) modifying the donor smooth muscle cells, or progenitors cells thereof, by inserting into the smooth muscle cell,

5   DNA encoding the A20 protein or mutant or derivative thereof; and (2) transplanting the resultant modified donor cells, or tissues or organs having such modified cells, into the recipient so that A20 is expressed in the transplanted cells, thereby substantially promoting apoptosis.

The donor species may be any species which is the same or

10   different from the recipient species, and which is able to provide the appropriate cells, tissues, or organs for transplantation into the recipient species. The donor may be of a species which is allogeneic or xenogeneic to that of the recipient. Preferably, the recipient is a mammal, e.g., a primate. Most preferably the recipient is human. For human recipients, it

15   is envisaged that human (i.e. allogeneic) as well as pig (i.e. xenogeneic) donors will be suitable, but any other mammalian species (e.g., bovine or non-human primate) may also be suitable as donors. For example, porcine aortic smooth muscle cells, or the progenitor cells thereof, can be modified to express porcine or human A20 protein at effective levels for grafting

20   into a human recipient.

Heterologous DNA encoding the A20 or another pro-apoptotic protein can be inserted into the animal or an ancestor of the animal at the single-cell stage or early morula stage. The preferred stage is the single-cell stage although the process may be carried out between the two and

25   eight cell stages. A transgenic animal can thereby be obtained, which will pass the heterologous DNA on to its offspring.

Methods of preparing transgenic pigs are included in Example 4. Methods of preparing transgenic pigs are well known in the art, as

discussed by Pinckert et al., *Xeno*, Vol. 2, No. 1, 1994 and the references cited therein, incorporated by reference herein. Of course, any transgenic animal may be used in the present invention. Pigs are particularly preferred because they are particularly amenable for xenotransplantation

5   into a human recipient. Preferably, the xenotransplanted tissue is in the form of an organ, for example, kidney, heart, or liver. Transgenic pigs may be produced by homologous recombination and other such techniques that destroy wild-type gene function. By way of example, transgenic pigs may be produced utilizing homologous recombination techniques to

10   produce a transgenic animal expressing the A20 protein, preferably the human A20 protein.

In another aspect, nucleic acid encoding the preferred genes can be inserted into somatic/body cells of the donor animal to provide a somatic recombinant animal from which the DNA construct is not capable of being

15   passed on to offspring (see, e.g., Miller, A.D. and Rosman, G.T., *Biotechniques* (1989) 7(9):980-990). Appropriate well-known methods of inserting foreign cells or DNA into animal tissue include, for example, micro-injection, embryonic stem cell manipulation, electroporation, cell gun, transduction, transfection, retroviral infection, adenoviruses, etc.

20   Prior to implantation into a recipient species, the treated smooth muscle cells, tissue or organ may be screened for modified cells containing and expressing the construct. For this purpose, the vector construct can also be provided with a second nucleotide sequence encoding an expression product that confers resistance to a selectable marker

25   substance. Suitable selection markers for screening include the neo gene, conferring resistance to neomycin or the neomycin analog, G418. Alternatively, a sequence encoding a tag, such as green fluorescent protein (GFP) or hemaglutinin A (HA), may be attached coding region of the A20

protein. Such tags are currently available and in use in our laboratory. Although any mammalian cell can be targeted for insertion of the anti-apoptotic gene, such as endothelial cells, monocytes, NK cells, lymphocytes, or islet cells, the preferred cells for manipulation are smooth

5   muscle cells. The recipient species will primarily be human, but not exclusively. Other mammals, such as non-human primates, may be suitable recipients.

In an alternative embodiment of the invention, the pro-apoptotic polypeptide, or mutant or derivative thereof, may be applied directly to

10   smooth muscle cells, tissues or organs *in vivo* in a phamaceutically acceptable carrier.

It will be appreciated that the modified donor smooth muscle cells and modified donor tissues and organs described above will be useful in the prevention of xenotransplant rejection (or delayed xenograft rejection

15   or acute vascular xenograft rejection) (see, Bach et al. *Immunology Today* (1996) 17:379-383; Dalmasso et al., *Transplantation* (1991) 52:530-533). Xenotransplant rejection accompanies the transplantation of organs between discordant species and involves an immediate immunologic response of recipient antibodies and the complement system against the

20   transplanted organ.

As described herein, inhibition of xenotransplant rejection may be achieved using the A20 protein of the present invention. This may be accomplished by providing transgenic animals that express the A20 protein of the recipient species. The smooth muscle cells of a donor organ

25   obtained from such an animal can be modified by any one of the gene therapy techniques defined above. Alternatively, a vector containing DNA encoding a protein having pro-apoptotic activity can be introduced into the transgenic animal at the single cell stage or early morula stage. In this

way, the resulting transgenic animal will express the complement
inhibitory factors and will have smooth muscle cells as defined above.
Thus, in a further aspect, the invention also provides smooth muscle cells,
tissue, donor organs, and non-human transgenic or somatic recombinant

5   animals, as defined above, which express one or more human complement
inhibitory factors.

It will be appreciated by those skilled in the art that the present
invention can be applied to prevent or treat a variety of vascular
conditions. As described herein, direct adenoviral-mediated transduction

10   of vessels can be used to treat ischemia/reperfusion injuries and
post-angioplasty of atherosclerotic plaques of both coronary and peripheral
arteries. In addition, as described above, venous grafts may also be
modified and used in coronary artery bypass graft surgery or for peripheral
re-vascularization of lower extremities (Mann et al., *supra*).

15   *Identifying Therapeutic Compounds*

Compound screening assays maybe used to identify bioactive
agents that are capable of inducing A20-mediated apoptosis of smooth
muscle cells in vascular lesion cells. Of particular interest are screening
assays for agents that induce A20-mediated apoptosis in smooth muscle

20   cells, yet have a low toxicity for other human cells. A wide variety of
assays may be used for this purpose, including labeled *in vitro* protein-
protein binding assays, electrophoretic mobility shift assays,
immunoassays for protein binding, and the like. Three-dimensional
crystal structures prepared from purified protein can be useful for

25   modeling intermolecular interactions, etc.

Accordingly, the present invention further provides a method of
identifying compounds that substantially promote apoptosis and

-33-

substantially inhibit smooth muscle cell growth or proliferation. The method includes the steps of (1) exposing smooth muscle cells to a test compound; and (2) assaying the smooth muscle cells for increased apoptosis and reduced growth or proliferation. For example, one could

5   assay for binding of the test compound compared to A20. Preferably, one could assay for activation of A20 activity. Generally a plurality of assay mixtures are run in parallel with different test compound concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e. at zero

10   concentration or below the level of detection.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of

15   randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means, and may

20   be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

The assay of the present invention may be a binding assay, wherein

25   one or more of the molecules may be joined to a label that can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like. Specific binding

-34-

molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule that is detectable in accordance with known procedures.

5   A variety of other reagents may be included in the binding assay. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc. that are used to facilitate optimal protein-protein binding and/or reduce nonspecific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors,

10   anti-microbial agents, etc. may also be used. The mixture of components is added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4° and 40° C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically

15   between 0.1 and 1 hours will be sufficient.

Alternatively, the well known yeast double-hybrid system can be used to define new protein-protein interactions (Young, *Biol. Reprod*, (1998) 58(2):302-311).

Drug Treatment

20   The present invention further provides a method of treating a patient having a vascularized organ transplant, a vessel transplant, or vascular disease by enhancing A20 biological activity. Once an agent has been identified, as described above, it may be used as a therapeutic agent for vascular disease. Thus, the present discovery can be used to identify

25   other potential therapeutic agents, including novel anti-atherogenic drugs. Alternatively, the present invention provides a method of preventing

vascular disease in a patient by enhancing the biological activity of A20.

Enhancing A20 biological activity may be accomplished, for example, by

administering to the patient a therapeutic agent. The therapeutic agent

may be any permeable factor such as a peptide or a protein, for example a

5   cytokine, or a drug, for example, a compound identified in the screening

method described above. Alternatively, the activity of A20 may be

enhanced by employing a mutant of the wild-type A20 gene that encodes

an A20 protein that displays increased biological activity. Measurement

of an increase in the biological activity of A20 includes assessing as to

10   whether there is a decrease in inflammation, an increase in apoptosis, or a

decrease in proliferation in smooth muscle cells expressing A20 protein

(see, Examples).

   In other preferred embodiments, the present invention provides a

method of promoting apoptosis in smooth muscle cells in a patient by

15   increasing the level of A20 in a patient's smooth muscle cells. Of course,

it will be appreciated that one mechanism by which the level of A20 may

be increased is by generating smooth muscle cells that express the A20

protein at a higher level than wild-type smooth muscle cells, as described

above. Another mechanism includes expressing a derivative or mutant of

20   the A20 gene that encodes an A20 protein that is expressed at a higher

level or has increased stability within the smooth muscle cell. Yet another

mechanism by which this embodiment is fulfilled is by administering, to a

patient, a therapeutic agent, as described above. In the present

embodiment, the therapeutic agent may be a transcription factor, for

25   example a transcription factor activated by an inflammatory reaction (e.g.,

a cytokine) that increases or stabilizes A20 expression in smooth muscle

cells. Alternatively, the therapeutic agent may be a compound that

induces increased expression or stability of the A20 protein.

-36-

One skilled in the art would appreciate that in order to assay for an increased level of A20 in a smooth muscle cell, using either a wild-type A20 protein or a mutant or derivative of A20, one would merely need to compare the quantity of A20 expression in modified and wild-type smooth

5 muscle cells (e.g., by semi-quantitative Western blotting or quantitative PCR). Likewise, one skilled in the art would appreciate that mutants and derivatives of the A20 protein can easily be identified by introducing mutations into the A20 protein and assaying the activity of the A20 protein in smooth muscle cells as described herein. Such an analysis is currently

10 being undertaken.

The compounds having the desired pharmacological activity may be administered, in a physiologically acceptable carrier, to a host for treatment of vascular disease. Depending upon the manner of introduction, the compounds may be provided in a variety of

15 physiologically acceptable carriers and formulations known to one skilled in the art and described, for example, in *Remington's Pharmaceutical Sciences*, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA, incorporated herein by reference. The concentration of the therapeutically active compound in the formulation may vary from about

20 0.1-100 weight %.

It will be appreciated that the therapeutic agents of the present invention may be coadministered with other agents. For example, the therapeutic agents of the present invention may be coadministered with an immunoregulatory factor (e.g., an immunostimulant, i.e., a cytokine, or an

25 immunosuppressant). Alternatively, a combination of drugs may be administered to a patient to induce a pro-apoptotic effect in smooth muscle

-37-

cells. Of course, any of the therapeutic agents described herein may be administered along with the A20 protein, or alternatively in conjunction with the overexpression of the A20 protein in a particular organ or tissue.

The therapeutic agents are administered at a dose effective to.
5   reduce the dimensions of a pre-existing intimal lesion. Usually the lesion will be reduced in size by at least about 20% over a period of one or more weeks, more usually by at least about 50%, and may be completely regressed.

In an alternative embodiment, the present invention provides a
10   method of treating or preventing vascular disease in a patient by preparing a smooth muscle cell, or tissue or organ comprising a smooth muscle cell, for transplant by treating the smooth muscle cell, or tissue or organ comprising a smooth muscle cell, with a therapeutic agent that increases A20 biological levels, and transplanting the smooth muscle cell, or tissue
15   or organ comprising the smooth muscle cell, treated with said therapeutic agent to a patient at risk for developing vascular disease. In a related embodiment, a therapeutic agent that increases the A20 level in the smooth muscle cells may be used to prepare the smooth muscle cell, or tissue, or organ for transplant. According to the present invention, the smooth
20   muscle cells, tissues or organs may be prepared *in vivo*, or *ex vivo*, as described herein above.

Examples

The present invention will now be illustrated by the following non-limiting examples. The following example demonstrate the anti-
25   inflammatory/anti-proliferative/pro-apoptotic effect of A20 in smooth muscle cells.

Example 1: A20 Activity in SMC

A20 is Part of the Physiological Response of SMC to TNF

Primary human aortic SMC were cultured in 6 well plates using smooth muscle basal medium (SmBM) (Clonetics, California)

5   supplemented with growth factors, Gentamycin and Amphotericin B. Confluent cells were stimulated with 100 Units of recombinant human (rhu)TNF and RNA was extracted before and at 1 and 6 hours (h) following addition of TNF. RNA samples were analyzed by Northern blot analysis for the expression of A20 using an A20 cDNA probe (Ferran et

10   al., *supra*). In all experiments, a cDNA probe for human GAPDH was used to evaluate equal loading of RNA. Results showed almost no A20 mRNA expression prior to TNF addition. A20 mRNA was strongly induced 1 h following TNF stimulation and started declining 6 h thereafter (Fig.1A). The induction of A20 by TNF was confirmed at the protein

15   level by means of immunohistochemistry using a rabbit anti-human A20 polyclonal antibody developed in the laboratory (Ferran et al., *supra*). A20 protein expression was detected in the cytoplasm of SMC 4 h following TNF treatment (Fig. 1B).

A20 Inhibits NF-κB-Activation in SMC

20   Vascular SMC, at sites of atherosclerotic lesions and TAV, express features of inflammatory processes, including increased expression of genes encoding growth factors and inducible surface proteins such as adhesion molecules, iNOS, CD40 and CD40L (Bourcier et al., *supra*; Mach et al., *supra*; Obrien et al., *Circulation* (1996) 93:672-682). NF-κB

25   has emerged as a regulator of most of these molecules by vascular cells. Inflammatory cytokines, oxidized lipids, activated monocytes, and T cells, all present in both human atheroma and TAV, activate NF-κB and elicit a

pro-inflammatory phenotype in SMC. In addition to the pro-inflammatory phenotype, activation of NF-κB is crucial for the acquisition by the SMC of the proliferative phenotype that leads to the formation of a neointima and thus development of arteriosclerotic lesions. We had previously

5   shown that A20 is a broad inhibitor of NF-κB activation in EC (Ferran et al., *supra*; Cooper et al., *J. Biol. Chem.* (1996) 271:18068-18073). We thus tested whether A20 retains this same function in SMC.

A20 was expressed in human SMC cultures using a recombinant A20 adenovirus (rAd.A20) (kind gift of Dr. V. Dixit). Recombinant

10 Ad.A20 was produced, purified, and titered as described earlier (Ferran et al., *supra*). In preliminary experiments, different multiplicities of infection (MOI), ranging from 100 to 1,000 plaque forming unit (pfu)/cell, were evaluated. Expression of A20 was tested by means of immunohistochemical analysis. Results show that high expression of A20

15 is achieved in 95% to 100% of the SMC infected with 500 pfu of rAd.A20/ cell without causing any cytotoxicity.

Nuclear and cytoplasmic extracts were prepared, before stimulation with TNF, at 15 minutes following stimulation with TNF, and at 2 h following stimulation with 100U/ml of TNF, from non-infected (non-

20 infected), rAd.A20 or rAd.β-gal-infected SMC. NF-κB activation and binding to specific kB elements were evaluated by means of electrophoretic mobility shift assay (EMSA). A20 expression in SMC inhibited NF-κB binding to DNA (Fig. 2A).

We also determined where the signaling pathway leading to NF-κB

25 activation is blocked by A20 in SMC. Cytoplasmic extracts were run on SDS-PAGE 12.5% minigels. IκBα was detected using a rabbit polyclonal IgG antibody (C21 from Santa Cruz) and a peroxidase-conjugated goat anti-rabbit secondary antibody followed by enhanced chemiluminescence

-40-

detection. A20 expression in SMC inhibited the usual IκBα degradation that occurs 15 minutes following TNF stimulation (Fig. 2B). These results demonstrate that A20 interferes with signaling pathways leading to NF-κB activation at a level upstream of IκBα degradation.

5   *Overexpression of A20 Inhibits the Up-Regulation of a NF-κB Dependent Genes in SMC*

ICAM1 was used as a marker of NF-κB trans-activating potential in SMC. The rationale for using ICAM-1 relies on its role in promoting accumulation of monocytes and lymphocytes that contribute to the

10   development of atherosclerotic lesions. Up-regulation of ICAM-1 following SMC stimulation was evaluated by Northern blot analysis using specific cDNA probes available in the laboratory. Overexpression of A20 inhibited the up-regulation of ICAM-1 mRNA induction in SMC following TNF treatment as compared to non-infected and

15   rAd.β-gal-infected cells (Fig.3). These results were confirmed at the protein levels.

Another NF-κB dependent gene that has been implicated in the development of atherosclerosis is the chemokine monocyte chemottractant protein (MCP)-1 (Ueda et al., *J. of Immunology* (1994) 153:2052-2063).

20   MCP-1 not only attracts monocytes to the site of vascular inflammation but has also been implicated in promoting SMC migration and proliferation in the neointima (Streblow et al., *Cell* (1999) pages 511-520). Like ICAM-1, induction of MCP-1 by pro-inflammatory cytokines is regulated at the transcription level. Up-regulation of MCP-1 following

25   TNF treatment of SMC was therefore evaluated by Northern blot analysis using a human cDNA probe as described (Millan et al., *Transplantation* (1997) 63:421-429). Over-expression of A20 in SMC inhibited TNF-

-41-

mediated up-regulation of MCP-1 mRNA as compared to not infected (NI) and rAd.β-gal-infected cells (Fig. 7).

Overexpression of A20 Inhibits SMC Proliferation

Having established that A20 inhibits NF-κB activation, we tested

5   whether such inhibition would affect the rate of SMC proliferation. Activation of NF-κB has been shown to be essential for SMC proliferation (Selzman et al., *Ann. Thorac. Surg.* (1999) 67:1227-1232). Cultured human SMC were plated at 50 to 60% confluence in 6 well plates and subsequently cultured in 0.5% FCS medium for 48 hours. SMC were kept

10 in the same medium and infected with rAd.A20, rAd. IκBα (kind gift of Dr. Christopher Wrighton), in addition to A20 to achieve NF-κB inhibition, or the control rAd.β-gal. Twenty-four hours following infection, the medium was replaced with 10% FCS and cells were recovered 24 and 48 h later for cell cycle analysis. DNA content was

15 analyzed by FACScan using Cellquest acquisition software. Analysis of DNA ploidy allows discrimination of cells in G0/G1 versus S versus G2/M phases of the cell cycle. Expression of A20 in SMC inhibited progression of the cells through the cell cycle. SMC expressing A20 are blocked in the G0/G1 phase of the cell cycle: 14.4% of the A20 expressing

20 SMC are in the S+G2/M phases of the cell cycle prior to serum addition. This percentage remains essentially the same at 24 and 48h (16%) following serum addition (Fig.4). In contrast, non-infected or rAd.β-gal-infected SMC proliferate in response to serum addition and progress to the S+G2/M phases of the cell cycle. The percentage of SMC

25 in S+G2/M phase of the cell cycle before and after serum addition are noted on Fig.4. SMC expressing the specific inhibitor of NF-κB, IκBα showed a similar pattern of cell cycle inhibition in response to serum as

the one obtained in A20 expressing cells. This latter data confirms that of

others showing that NF-κB activation is required for SMC proliferation

and is blocked by inhibitors of NF-κB (Autieri et al., *Biochem. Bioph. Res.*

Com. (1995) 213:827-836; Selzman et al., *supra*). It also suggests that the

5   inhibitory potential of A20 upon SMC proliferation relates to its ability to

block NF-κB activation.

Overexpression of A20 Sensitizes SMC to Cytokine- and Fas-Mediated
Apoptosis

SMC are highly resistant to apoptosis. The molecular basis of this

10 resistance is poorly defined. SMC are readily induced to undergo

apoptosis in culture when stimulated with high doses of cytokines

including interferon-γ, TNF, and IL-1β, deprived of growth factors, or

exposed to oxidized LDL, NO donors or the anti-oxidant pyrrolidine

dithiocarbamate (PDTC). We thus tested whether A20, a well accepted

15 anti-apoptotic gene in EC, B cells, and fibroblasts also has this function in

SMC. Non-infected, rAd.A20, rAd.β-gal or rAd.IκBα infected SMC were

treated with 400 U/ml of rhuTNF, 400 U/ml rhu IFNγ and 100 U/ml of rhu

IL-1β/ml. Forty-eight hours following treatment, cells were harvested,

and apoptosis evaluated by DNA content analysis (Ferran et al., *supra*).

20 Cells with a normal DNA content (>2N) were scored as viable, whereas

cells with a hypodiploid DNA content (<2N, termed A0) were scored as

apoptotic. Surprisingly, our results revealed that expression of A20 in

SMC sensitizes them to cytokine-mediated apoptosis. The percent of

apoptotic cells following cytokine treatment increases from 0.3% to 1.6%

25 in non-infected cells, and from 1.2% to 7% in the rAd.β-gal-infected SMC

(Fig.5). In contrast, the percentage of A20-expressing SMC undergoing

apoptosis following cytokine treatment increased dramatically and reached

-43-

36% at 48h. Interestingly, expression of IκBα in SMC sensitized the SMC to cytokine-mediated apoptosis, but not to the same extent as A20 despite equivalent inhibition of NF-κB by A20 and IκBα. The percentage of apoptotic SMC expressing IκBα increased from 0.8% to 16% 48h

5   following cytokine treatment. This data suggests that A20-induced sensitization to cytokine-mediated apoptosis is not solely related to its NF-κB inhibitory activity (Fig.5).

Another apoptotic receptor that is expressed on vascular SMC is Fas (CD95) (Chan et al., *Circ. Res.* (2000) 86:1038-1046). However and

10   despite Fas expression, many cells including SMC resist Fas-induced apoptosis. Interestingly, medial SMC show a marked heterogeneity to Fas-mediated apoptosis with their sensitivity determined not only by surface expression of Fas but also by differential expression of Fas-signaling proteins below receptor level such as caspase 3 (Chan et al.,

15   *supra*). Interestingly, Fas-mediated apoptosis of smooth muscle cells (SMC) has been suggested as a potential means to inhibit neointima formation (Sata et al., *Proc. Natl. Acad. Sci. USA* (1998) 95:11213-1217). Fas ligand gene transfer to the vessels inhibits neointima formation. We thus questioned whether A20-mediated sensitization to cytokine-mediated

20   apoptosis would also apply for Fas-mediated apoptosis. Non-infected, rAd.A20 or rAd.β-gal infected SMC were treated with 1 μg/ml anti-Fas antibody IgM clone CH-11 (αFas) (05-201 Upstate or an IgM control antibody in the presence or absence of Actinomycin D (150 nM). Sixteen hours following treatment, cells were harvested, and apoptosis evaluated

25   by DNA content analysis as described earlier. Cells with a normal DNA content (>2N) were scored as viable, whereas cells with a hypodiploid DNA content (<2N, termed A_0) were scored as apoptotic. Our results revealed that expression of A20 in SMC sensitizes them to α-Fas-mediated

apoptosis. The percent of apoptotic cells following αFas treatment
increases from 0.34% to 1.9% in NI cells, and from 0.7% to 2.34% in the
rAd.β-gal-infected SMC (Fig. 8). In contrast, the percentage of A20-
expressing SMC undergoing apoptosis following cytokine treatment

5   increased dramatically and reached 33% at 16 hours (Fig. 8). Killing was
specific for the αFas antibody as control IgM treatment did not modify the
percentage of apoptosis in these cells. Interestingly, pretreatment of EC
with Actinomycin D did not significantly modify the percentage of αFas-
induced apoptosis in SMC whereas it rendered NI and rAd.β-gal infected

10   SMC more prone to apoptosis with the percentage of apoptotic cells
reaching the same level as that in A20 expressing SMC (28-35%) (Fig. 8).
These results extend the sensitizing function of A20 to apoptosis in SMC
to a clearly relevant stimulus within the atherosclerotic lesion i.e. Fas.

The above-mentioned, rather unexpected, results contrast with the

15   anti-apoptotic function of A20 in EC and other cells. We had previously
shown that A20 protects EC from cycloheximide/TNF induced apoptosis
and can even overcome IκBα-mediated sensitization of these cells to
TNF-induced cell death (Ferran et al., *supra*).

Expression of A20 in SMC Inhibits αFAS-Mediated Activation of SMC

20   Having shown that A20 sensitizes SMC to αFas-mediated
. apoptosis, we were concerned about its impact upon the recently described
Fas/FADD-mediated activation of inflammatory gene expression in
smooth muscle cells. Indeed, Schaub et al have shown that signals
initiated by Fas-associated death doman protein (FADD) in SMC induce

25   the expression of chemokines such as MCP-1 and IL-8 and cause massive
immigration of macrophages *in vivo* at the site of atherosclerotic lesions
(Schaub et al., *Nature Medicine* (2000) 6:790-796). These chemokines are

also up-regulated during Fas-induced apoptosis in SMC. Induction of a pro-inflammatory program in SMC during Fas-mediated activation and apoptosis could be deleterious and contribute to the pathogenesis of vascular disease, a side-effect that is clearly undesirable if one is aiming to

5   inhibit atherosclerosis. We therefore checked whether expression of A20 in SMC would, as with the cytokines, inhibit Fas-mediated activation of NF-κB and up-regulation of MCP-1. SMC were either NI or infected with rAd.A20. Forty eight hours following infection, SMC were stimulated with 1 μg/ml of αFas antibody and total RNA was extracted at 0 and 4

10 hours after stimulation. MCP-1 expression following αFas treatment was analyzed by Northern blot analysis using a human MCP-1 probe. Results confirm that MCP-1 mRNA is up-regulated in SMC 4 hours following αFas addition. Overexpression of A20 in SMC inhibited αFas-mediated up-regulation of MCP-1 mRNA and also down-regulated the constitutive

15 level detected in NI cells (Fig. 9).

A20-Mediated Sensitization of SMC to Cytokines Is not Associated with a Modification in the Level of the Two Prototypic Anti-Apoptotic BCL Family Members BCL-2 and BCL-X_L

Given the sensitizing effect of A20 expression in SMC upon

20 cytokine and Fas-mediated apoptosis, we questioned whether expression of A20 was modifying the level of expression of Bcl-2 and Bcl-x_L. SMC were either NI or infected with rAd.A20 and rAd.β-gal as described earlier. Forty eight hours following infection, SMC were stimulated with TNF, IL-1β and IFNγ for 16 hours.

25   Cells were then trypsinized washed with PBS and subsequently lysed in RIPA Buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Deoxycolate and 1% SDS) supplemented with protease inhibitors

(1μM of aprotinin and leupeptin, 0.1μM of TLCK and TPCK and 0.5 μM of PMSF). Protein concentration was measured using Bio-RAD D_c protein assay (Bio-Rad, Hercules, CA) and fifteen μg of protein were resolved on reducing sodium dodecyl sulfate (SDS) polyacrylamide gels,

5 transferred onto Immobilon-P membranes (Milipore, Bedford, MA) and blocked in Blotto (5% non-fat dry milk in 0.1% Tween 20 in PBS). Bcl-$x_{S/L}$ and Bcl-2 were detected using 1/500 dilution of an anti Bcl-$x_{S/L}$ rabbit IgG (Santa Cruz) and a 1/1000 dilution of an anti-Bcl-2 mouse IgG1 mAb (Pharmingen, San Diego, CA) followed by a 1/3000 dilution of

10 peroxydase-conjugated donkey anti-rabbit or goat anti-mouse secondary antibody (Pierce, Rockford, IL) and revealed by enhanced chemiluminescence (NEN, Boston, MA). Results indicate that expression of A20 in SMC does not modify the constitutive levels of expression of Bcl-x_L nor their levels upon addition of cytokines (Fig. 10).

15 *Establishment of a Mouse Line Expressing A20 in EC*

A transgenic mouse line expressing A20 in EC was established using the Complement (C)1 inhibitor promoter that has been shown to induce specific expression of the transgene in EC (Dr. Mario Tozzi, personal communication). Screening for expression of A20 in EC is

20 currently being performed to establish a line of high expressers. Analysis of A20 expression by means of immunohistochemistry shows specific expression of A20 in EC of the heart (Fig.6 A&B).

Example 2: Evaluation of the Effects of Expressing A20 in SMC _In Vitro_
on Inhibition of NF-κB Activation and the Impact on SMC Activation and
Proliferation

Induction of A20

5   One may determine whether A20 expression is part of the
physiological response of the SMC to injury.

Fourth to sixth passage human aortic SMC monolayer cultures are
activated using different agonists that are relevant to the pathogenesis of
atherosclerosis. These agonists include TNF, LDL, and oxidized LDL,

10   growth factors (platelet derived growth factor (PDGF)) and CD40
cross-linking. The expression of A20 in SMC following these agonists is
evaluated at different time points (1-24 h), both at the mRNA and protein
levels as described in the preliminary results. One then tests whether each
of these critical mediators of the atherosclerotic lesion will, like TNF,

15   induce the expression of A20. We propose, without limiting the
biochemical mechanism of the invention, that A20 induction is part of the
"regulatory" response of SMC to injury. Expression of A20 in the SMC
may result in the prevention of neointimal hyperplasia. It is presumably
only when these regulatory mechanisms are overwhelmed that

20   atherosclerosis develops.

A20 Overexpression

One may also test the effect of A20 overexpression via
adenoviral-mediated gene transfer in SMC upon (i) NF-κB activation, (ii)
up-regulation of NF-κB dependent genes such as ICAM1, inducible nitric

25   oxide synthase (iNOS), microphage chemoattractant factor 1(MCP1) and
CD40 (all involved in promoting atherosclerosis) (iii) SMC proliferation
and cell cycle regulation. To determine whether the effect of A20 on

NF-κB activation is transcription factor specific, one may evaluate whether expression of A20 in SMC affects other transcription factors or signaling pathways involved in SMC proliferation and apoptosis. These include nuclear factor for activated T cells (NF-AT), activated protein-1

5   (AP-1) and the mitogen -activated protein kinase (MAPK) signaling pathways.

Mechanisms of Action of A20 in SMC Upon NF-κB Activation

The central role of NF-κB activation in the development of atherosclerosis is discussed above. The experiments described here are

10   aimed at confirming and extending, to other pro-atherogenic stimuli, our data showing that overexpression of A20 inhibits TNF-mediated NF-κB activation and NF-κB dependent gene expression in SMC. In addition, one may dissect further the level at which A20 interferes within the signaling pathway leading to NF-κB activation.

15   SMC cultures overexpressing A20 by means of adenoviral-mediated gene transfer may be activated using oxidized LDL or CD40 cross-linking, two relevant pro-atherogenic stimuli. As discussed above, TNF has already been tested. Whole cells, cytoplasmic and nuclear extracts, and mRNA are harvested at different time points following these

20   treatments and evaluated for (i) expression of the NF-κB dependent genes ICAM-1, VCAM-1, iNOS and CD40, all implicated in the pathogenesis of atherosclerosis; (ii) degradation and phosphorylation of IκBα by means of Western blot analysis; (iii) nuclear translocation of NF-κB by means of EMSA; and (iv) activation of the different kinases (IKKα, IKKβ, NIK and

25   MEKK1) involved in the signaling pathway leading to IκBα

phosphorylation (Zandi et al., *Mol. Cell. Biol.* (1999) 19:4547-4551).. The effect of A20 on these kinases is tested either by means of specific antibodies recognizing the phosphorylated forms of these kinases or by means of *in vitro* kinase assay evaluating the activity of these kinases on

5   their specific substrates.

The effect of A20 may also be evaluated on NF-AT and p38 MAP kinase activation to study their potential roles in SMC proliferation and apoptosis (Jing, et al., *Circ. Res.* (1999) 84:831-839; Boss et al., *J. Biol. Chem.* (1998) 273:19664-19671). This study is performed using EMSA

10 and kinase assays. The results obtained with A20 expressing SMC are then compared to those obtained with either non-infected, rAd.β-gal or rAd.IκBα-infected SMC control groups.

We believe that, as in EC, A20 will act in an agonist independent manner as a broad inhibitor of SMC activation and that this inhibition will

15 occur upstream of IκBα degradation and phosphorylation. One may determine the exact level at which A20 interferes within the kinase cascade leading to NF-κB activation and compare these results with our data obtained in EC. A further delineation of a specific target of A20 in SMC provides data valuable to defining novel therapeutic strategies.

20 These data will also determine to what extent the effect of A20 in SMC is specific to NF-κB or affects other transcription factors or signaling pathways relevant for SMC proliferation and apoptosis.

The Effect of A20 Expression Upon SMC Proliferation

SMC proliferation is another marker of NF-κB activation. These

25 experiments test whether by blocking NF-κB activation, A20 will also inhibit SMC proliferation. As described above, SMC cultures are first serum-starved to synchronize their cell cycle within the G0 phase. SMC

-50-

are then infected with rAd.A20, rAd.IκBα or rAd.β-gal. Entry into the
cell cycle and proliferation are then driven by addition of serum or basic
fibroblast growth factor (bFGF). Cell cycle progression is then evaluated
by means of FACS analysis of DNA content and bromodeoxyuridine

5   incorporation at 24, 48, 60 and 72 h following addition of the growth
factors. In addition, cell extracts and mRNA are recovered following the
addition of serum to evaluate the effect of A20 expression on cyclin D1, a
critical NF-κB-dependent component of the cell cycle. We propose,
without limiting the invention, that overexpression of A20 in SMC is

10   likely to be at least as effective as IκBα in inhibiting the proliferation of
SMC, with the SMC being retained within the G1 phase of the cell cycle.
We believe this effect relates to inhibition of the NF-κB-dependent
expression of cyclin D1.

                    Example 3: A20 Activity *In Vivo* and *In Vitro*

15   *Impact of A20 Overexpression Upon SMC Apoptosis In Vitro*
            Apoptosis is now viewed as beneficial to prevent redevelopment
and promote the regression of established atherosclerotic lesions. The
present invention demonstrates for the first time that A20 sensitizes SMC
to cytokine-mediated apoptosis, making A20 a prime gene therapy target

20   to achieve this aim. These experiments are planned to extend our finding
that A20 sensitizes SMC to cytokine-mediated apoptosis and to other
apoptotic stimuli that are present within the atherosclerotic plaque i.e. Fas,
NO and oxidized LDL. Second, one may determine the molecular basis of
the effect of A20 upon the death signaling machinery. These studies

25   evaluate the effect of A20 on activation of caspases, mitochondrial
membrane potential, c-myc, cytochrome c release and cleavage of death
substrates such as PARP that are the hallmarks of apoptosis.

As described above, non-infected SMC and SMC infected with rAd.A20, rAd.IκBα or rAd.β-gal are subjected to 4 different pro-apoptotic stimuli, i.e. a combination of TNF, IL-1 and IFNγ, NO donors, oxidized LDL and Fas cross-linking with anti-Fas antibodies. Apoptosis is assayed

5   at 8, 16, 24 and 48 h following stimulation by means of FACS analysis of
 · DNA content. In addition, cell extracts are recovered at 3, 6, 9, 16 and 24
 h to analyze (i) the effect of A20 on the activation of both initiator or class
 I (caspase 8) and effector or class II (caspase 3,9) caspases using specific
 colorimetric assays and Western blot analysis, (ii) cytochrome c release in

10   the cytoplasm (iii) c-myc expression and (iv) cleavage of PARP. The
 effect of A20 on modification of the trans-mitochondrial membrane
 potential will also be evaluated.

We have demonstrated that although anti-apoptotic in EC and other cells, A20 functions to sensitize SMC to TNF-mediated apoptosis and can

15   thus contain abnormal cell growth and proliferation. This seems
 especially true in an inflammatory environment (which induces expression .
 of A20) that is encountered in rejecting allografts and in lesions of
 atherosclerosis.

As noted above, this effect relates to more than A20-mediated

20   inhibition of
 NF-κB, because the sensitizing effect of A20 upon apoptosis is stronger
 than the one achieved by IκBα despite a similar blockade of NF-κB
 activation by A20 and IκBα. Evaluating whether A20 has additional
 effects on other signaling pathways involved in SMC apoptosis such as

25   p38 MAP kinase will further determine the mechanisms by which A20
 sensitizes SMC to apoptosis. This novel function of A20 in SMC may
 indicate specific molecular partners for A20 in SMC. One may identify
 such specific molecular partners by using a yeast double-hybrid system

using A20 as a bait and a SMC cDNA library. These experiments will allow the identification of new partners for A20, in addition to those already described, i.e., TNF receptor-associated factor (TRAF)-1,2 and 6, 14-3-3 and A20 binding inhibitor of NF-κB (ABIN) (Vincenz et al., *J.*

5 *Biol. Chem.* (1996) 271:20029-20034; Heynink et al., *FEBS Lett.* (1999) 442:147-150; Heyninck et al., *J. Cell Biol.* (1999) 145:1471-1482; Yeong-Song et al., *Proc. Natl. Acad. Sci. USA* (1996) 93:6721-6725).

The Anti-atherogenic Potential of A20 In Vivo

Both our *in vitro* and *in vivo* data provide a rationale for using A20

10 to counter the development of atherosclerosis. A20 functions *in vitro* to inhibit SMC activation and proliferation and sensitizes SMC to cytokine-mediated apoptosis. *In vivo*, A20 expression in SMC of long term surviving heart xenografts and allografts correlates with the absence of atherosclerosis. We propose that A20 has strong anti-atherogenic

15 potential.

Two different models may be used. First, the well established model of balloon injury of carotid arteries in rabbits (Pollman et al., *supra*, Guzman et al., *Circulation* (1993) 88:2838-2848). This model enables one to study the effect of adenoviral-mediated gene transfer of A20 to the

20 vessel wall (adenoviral-mediated gene transfer to vessels achieves transient but high levels of transgene expression (Guzman et al., *supra*)) in protecting from intimal hyperplasia, restenosis and atherosclerosis development when the animal are fed a high cholesterol diet.

Second, one may utilize a transgenic mouse expressing A20 in

25 SMC under the control of one of the newly-developed SMC promoters: the Crp2/SmLim promoter or the smooth muscle heavy chain myosin gene promoter (Madsen et al., *Circ. Res.* (1998) 82:908-917,;Yet et al., *J. Biol.*

Chem. (1998) 273:10530-10537). Of course, a variety of promoters known in the art are also suitable for use in this experiment. A20 is cloned under the control of these promoters and the mice are screened for the presence of the transgene by Southern blot analysis and for expression in

5 vessels by immunohistochemistry. These mice are then used as organ donors in models of chronic allograft or xenograft rejection (Hancock et al., *supra*; Bach et al., *Transpl. Proc.* (1997) 29:56-58).

Additionally, these mice may be crossed with mice expressing A20 in EC to test whether expression of A20 is required in both EC and SMC

10 to inhibit TAV. Mice expressing A20 in their SMC or in their SMC and EC are then crossed with already established lines of atherosclerosis prone mice (i.e., the LDL receptor knock out or the ApoE deficient mice), to evaluate whether A20 will protect these animals from the development of atherosclerotic lesions (Breslow, *Science* (1996) 272:685-688). Two

15 transgenic mouse lines that may be used are the LDL-receptor deficient mice available through the Jackson Laboratory and the APO E deficient mice (Plump et al., *Cell* (1992) 71:343-352; Ishibashi et al., *J. Clin. Invest.* (1994) 93:1885-1893). The evaluation of the anatomical lesions and their correlation with the expression of the transgene may be performed by

20 immunohistochemistry.

We predict that gene transfer of A20 to the vessel wall will inhibit intimal proliferation and restenosis in the rabbit balloon injury model. Based upon this, clinical therapies using adenoviral or lentiviral vectors may be developed (Morsy et al., *Proc. Natl. Acad. Sci.* (1998) 95:7866-

25 7871; Amado et al., *Science* (1999) 285:674-676). Generation of mice expressing A20 in SMC may be done to confirm the protective function of A20 when expressed in SMC (and EC) in protecting from TAV. Conditional expression of the transgene under the control of the

-54-

tetracycline operon may be needed to circumvent these potential results. Such constructs are publicly available (Gossen et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:5547-5551).

Example 4: Preparation of Transgenic Mammals

5   Recombinant constructs are well known in the art, (see, Maniatis et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y.). Methods of producing transgenic mammals using recombinant constructs are well known and include, but not limited to, microinjection, embryonic stem (ES) cell manipulation, electroporation, cell gun,

10   transfection, transduction, retroviral infection, etc. Species of constructs may be introduced individually or in groups of two or more types of constructs.

According to one preferred embodiment of the invention, a transgenic pig may be produced that expresses a SMC specific pro-

15   apoptotic factor (e.g., A20). Briefly, estrus may be synchronized in sexually mature gilts (>7 months of age) by feeding an orally active progestogen (allyl trenbolone, AT: 15 mg/gilt/day) for 12 to 14 days. On the last day of AT feeding all gilts may be given an intramuscular injection (IM) of prostaglandin F_{2a} (Lutalyse: 10 mg/injection) at 0800 and 1600

20   hours. Twenty-four hours after the last day of AT consumption all donor gilts may be administered a single IM injection of pregnant mare serum gonadotropin (PMSG: 1500 IU). Human chorionic gonadotropin (HCG: 750 IU) may be administered to all donors at 80 hours after PMSG.

Following AT withdrawal, donor and recipient gilts may be

25   checked twice daily for signs of estrus using a mature boar. Donors that

exhibit estrus within 36 hours following HCG administration may be bred at 12 and 24 hours after the onset of estrus using artificial and natural (respectively) insemination.

Between 59 and 66 hours after the administration of HCG one- and
5   two-cell ova may be surgically recovered from bred donors using the following procedure. General anesthesia may be induced by administering 0.5 mg of acepromazine/kg of body weight and 1.3 mg ketamine/kg of body weight via a peripheral ear vein. Following anesthetization, the reproductive tract may be exteriorized following a mid-ventral
10   laparotomy. A drawn glass cannula (O.D. 5 mm, length 8 cm) may be inserted into the ostium of the oviduct and anchored to the infundibulum using a single silk (2-0) suture. Ova may be flushed in retrograde fashion by inserting a 20 g needle into the lumen of the oviduct 2 cm anterior to the uterotubal junction. Sterile Dulbecco's phosphate buffered saline
15   (PBS) supplemented with 0.4% bovine serum albumin (BSA) may be infused into the oviduct and flushed toward the glass cannula. The medium may be collected into sterile 17X100 mm polystyrene tubes. Flushings may be transferred to 10X60 mm petri dishes and searched at low power (50X) using a Wild M3 stereomicroscope. All one- and
20   two-cell ova may be washed twice in Brinster's Modified Ova Culture-3 medium (BMOC-3) supplemented with 1.5% BSA and transferred to 50 μl drops of BMOC-3 medium under oil. Ova may be stored at 38°C. under a 90% N_2, 5% O_2, 5% CO_2 atmosphere until microinjection is performed.

One- and two-cell ova may be placed in an Eppendorf tube (15 ova
25   per tube) containing 1 ml HEPES Medium supplemented with 1.5% BSA and centrifuged for 6 minutes at 14000Xg in order to visualize pronuclei in one-cell and nuclei in two-cell ova. Ova may then be transferred to a 5-10 μl drop of HEPES medium under oil on a depression slide. Microinjection

may be performed using a Laborlux microscope with Nomarski optics and
two Leitz micromanipulators and 10-1700 copies of construct DNA
(linearized at a concentration of about 1 ng/μl of Tris-EDTA buffer) may
be injected into one pronuclei in one-cell ova or both nuclei in two-cell

5   ova.

Microinjected ova may be returned to microdrops of BMOC-3
medium under oil and maintained at 38°C. under a 90% N_2, 5% CO_2, 5%
O_2 atmosphere prior to their transfer to suitable recipients. Ova may
preferably be transferred within 10 hours of recovery.

10   Only recipients which exhibit estrus on the same day or 24 hours
later than the donors may preferably be utilized for embryo transfer.
Recipients may be anesthetized as described earlier. Following
exteriorization of one oviduct, at least 30 injected one-and/or two-cell ova
and 4-6 control ova may be transferred in the following manner. The

15   tubing from a 21 g X 3/4 butterfly infusion set may be connected to a 1 cc
syringe. The ova and 1-2 mls of BMOC-3 medium may be aspirated into
the tubing. The tubing may then be fed through the ostium of the oviduct
until the tip reaches the lower third or isthmus of the oviduct. The ova
may be subsequently expelled as the tubing is slowly withdrawn.

20   The exposed portion of the reproductive tract may be bathed in a
sterile 10% glycerol-0.9% saline solution and returned to the body cavity.
The connective tissue encompassing the linea alba, the fat, and the skin
may be sutured as three separate layers.

An uninterrupted Halstead stitch may be used to close the linea alba. The
25   fat and skin may be closed using a simple continuous and mattress stitch,
respectively. A topical antibacterial agent (e.g. Furazolidone) may then be
administered to the incision area.

Recipients may be penned in groups of about four and fed 1.8 kg of

a standard 16% crude protein corn-soybean pelleted ration. Beginning on

day 18 (day 0=onset of estrus), all recipients may be checked daily for

signs of estrus using a mature boar. On day 35, pregnancy detection may

5   be performed using ultrasound. On day 107 of gestation recipients may be

transferred to the farrowing suite. In order to ensure attendance at

farrowing time, farrowing may be induced by the administration of

prostaglandin F_{2a} (10 mg/injection) at 0800 and 1400 hours on day 112 of

gestation. In all cases, recipients may be expected to farrow within 34

10   hours following PGF2a administration.

Twenty-four hours after birth, all piglets may be processed, i.e. ears

notched, needle teeth clipped, 1 cc of iron dextran administered, etc. A

tail biopsy and blood may also be obtained from each pig.

For additional methods for making transgenic pigs see, Pinckert et

15   al., *Xeno*, Vol. 2, No. 1, 1994, incorporated herein by reference. One

skilled in the art would, of course, know how to modify these methods

from the exact protocols disclosed herein.

Summary

The results above demonstrate a novel function for A20 in SMC:

20   inhibition of SMC proliferation and sensitization to apoptosis. This later

function contrasts with the cytoprotective effect of A20 in EC and makes

A20 a unique gene therapy tool to protect the vessels from the

development of atherosclerosis. Indeed, the conventional paradigm

emphasizes the critical role of EC activation and SMC proliferation as two

25   culprits of atherosclerotic lesion progression. The present invention

provides evidence that A20 can function to inhibit both of these deleterious components, in part based on the pro-apoptotic effects of A20 in SMC.

The cellular and molecular biology methods that we intend to use in 5 the experiments described above are well known in the art. This includes experimental models of mouse transplantation. The balloon injury model will be carried out under the guidance of surgeons expert in the technique within the NIH-funded Harvard-Longwood Research Training in Vascular Surgery, with which we are associated.

10 All references cited herein are hereby incorporated by reference in their entirety.

What is claimed is:

1. A method of treating or preventing vascular disease comprising modifying a mammalian smooth muscle cell by inserting into said smooth muscle cell, or a progenitor thereof, DNA encoding a protein capable of decreasing inflammation and increasing apoptosis of said smooth muscle

5   cell and transplanting said smooth muscle cell, or a tissue or organ comprising such a cell, into a transplant candidate or a patient diagnosed with a vascular disease, wherein said protein has A20 activity.

2. The method of claim 1, wherein said vascular disease comprises atherosclerosis, transplant-associated vasculopathy, or chronic rejection.

10   3. A method of treating or preventing transplant-associated vasculopathy (TAV) comprising modifying a mammalian smooth muscle cell by inserting into said smooth muscle cell, or a progenitor thereof, DNA encoding a protein capable of decreasing inflammation and increasing apoptosis of said smooth muscle cell and transplanting said

15   smooth muscle cell, or a tissue or organ comprising such a cell into a patient at risk for developing TAV, wherein said protein has A20 activity.

4. The method of claim 1 or 3, wherein said protein is A20.

5. The method of claim 1 or 3, wherein said protein further blocks smooth muscle cell proliferation.

20   6. The method of claim 4, wherein said A20 comprises a mutant or derivative of said A20.

7. A method of treating atherosclerosis comprising transplanting donor smooth muscle cells, or graftable tissues or organs comprising smooth muscle cells, to a mammalian recipient having atherosclerosis or a vascularized organ transplant, comprising the steps of (a) modifying the

5   donor smooth muscle cells, or progenitor smooth muscle cells thereof, by inserting therein DNA encoding A20; and (b) transplanting the resultant modified donor smooth muscle cells, or tissues or organ comprising the cells into the recipient, and expressing in the cells A20, thereby substantially promoting apoptosis.

10   8. A method of preventing TAV comprising, transplanting donor smooth muscle cells, or graftable tissues or organs comprising smooth muscle cells, to a mammalian recipient comprising the steps of (a) modifying the donor smooth muscle cells, or progenitor smooth muscle cells thereof, by inserting therein DNA encoding A20; and (b)

15   transplanting the resultant modified donor smooth muscle cells, or tissues or organ comprising the cells into the recipient, and expressing in the cells A20, thereby substantially promoting apoptosis in the presence of a cellular activating stimulus.

9. The method of claim 7 or 8, wherein said method substantially
20   inhibits inflammation in the presence of a cellular activating stimulus.

10. The method of claim 7 or 8, wherein said A20 comprises a mutant or derivatives of A20.

11. A method of preparing a vascularized organ or vessel for transplantation into a patient comprising obtaining an organ or vessel for transplant and perfusing said organ or vessel with nucleic acid encoding A20 protein.

5   12. The method of claim 11, wherein said nucleic acid encoding A20 is inserted within a vector comprising an adenovirus vector or a lentivirus vector.

13. The method of claim 11, further comprising perfusing said organ or vessel with an immunoregulatory factor.

10   14. The method of claim 13, wherein said immunoregulatory factor is a cytokine.

15. The method of claim 13, wherein said immunoregulatory factor is Fas.

16. The method of claim 13, wherein said perfusing occurs *in vivo*.

15   17. The method of claim 13, wherein said perfusing occurs *ex vivo*.

18. The method of claim 12, wherein said A20 comprises a mutant or derivative of said A20.

19. The method of claim 11, further comprising enhancing the expression of A20 using a therapeutic agent.

20. The method of claim 11, wherein said patient has suffered ischemia, reperfusion injury, mechanical injury, immunologic injury, pharmacologic injury, or coronary trauma.

21. The method of claim 11, wherein said patient has undergone

5   balloon angioplasty.

22. The method of claim 11, wherein said patient has atherosclerosis.

23. The method of claim 11, wherein said organ or vessel is being prepared as a vascularized organ or a vascular graft.

10   24. A mammalian smooth muscle cell which is modified to express A20, said A20 being capable of inhibiting inflammation and promoting apoptosis in the presence of a cellular activating stimulus, said modified smooth muscle cell exhibiting increased sensitivity to apoptosis, relative to an untreated smooth muscle cell.

15   25. The smooth muscle cell of claim 24, wherein said A20 further blocks smooth muscle cell proliferation.

26. The smooth muscle cell of claim 24, wherein said A20 comprises a mutant or derivative of A20.

27. A donor smooth muscle cell, or a tissue or organ comprising such a cell, for transplantation into a recipient species, wherein said cell is modified to express A20, said A20 being capable of inhibiting inflammation and promoting apoptosis in the presence of a cellular

5   activating stimulus in said recipient.

28. The smooth muscle cell of claim 27, wherein said A20 further blocks smooth muscle cell proliferation.

29. The smooth muscle cell of claim 27, wherein said A20 comprises a mutant or derivative of A20.

10   30. An organ or vessel from a non-human transgenic or somatic recombinant mammal comprising DNA encoding a pro-apoptotic protein of a different species, wherein said protein has A20 activity.

31. An organ or vessel according to claim 30 which is porcine.

32. An organ or vessel according to claim 30, wherein said pro-
15   apoptotic protein is human.

33. A method of identifying compounds that substantially promote apoptosis and substantially inhibit growth or proliferation in smooth muscle cells comprising,
         a) exposing smooth muscle cells to a compound; and
20   b) measuring activation of A20 in smooth muscle cells.

34. A method of identifying compounds that substantially promote apoptosis and substantially inhibit growth or proliferation in smooth muscle cells comprising,

a) exposing smooth muscle cells to a compound; and

5   b) measuring binding of said compound to a pro-apoptotic protein, wherein said protein is A20.

35. A method of treating a patient having a vascularized organ transplant, a vessel transplant, or vascular disease comprising enhancing A20 biological activity.

10   36. A method of preventing vascular disease in a patient comprising enhancing A20 biological activity.

37. The method of claim 35 or 36, wherein said method comprises administering to said patient a therapeutic agent.

38. The method of claim 37, wherein said therapeutic agent
15 comprises a cytokine.

39. The method of claim 37, wherein said therapeutic agent comprises Fas.

40. The method of claim 37, wherein said therapeutic agent comprises a drug.

41. A method of promoting apoptosis in smooth muscle cells in a patient, said method comprising increasing the level of A20 in said smooth muscle cells.

42. The method of claim 41, wherein said smooth muscle cell is engineered to express A20.

43. The method of claim 36 or 42, wherein said A20 protein comprises a mutant or derivative of said A20 protein.

44. The method of claim 41, wherein said method comprises administering to said patient a therapeutic agent.

45. The method of claim 44, wherein said therapeutic agent comprises a transcription factor.

46. The method of claim 44, wherein said therapeutic agent comprises a cytokine.

47. The method of claim 44, wherein said therapeutic agent comprises a drug.

48. The method of claim 44, wherein said therapeutic agent comprises Fas.

49. The method of claim 41, wherein said patient has a vascularized organ transplant, a vessel transplant, or vascular disease.

50. The method of claim 49, wherein said vascularized organ comprises the heart, liver, or kidney.

51. The method of claim 49, wherein said vascular disease comprises atherosclerosis or TAV.

5   52. A method of inducing cell death of undesired proliferating smooth muscle cells within the neointima of a diseased vessel, said method comprising, enhancing A20 biological activity in said smooth muscle cell.

53. A method of inducing cell death of undesired proliferating

10   smooth muscle cells within the neointima of a diseased vessel, said method comprising, increasing the level of A20 in said smooth muscle cell.

54. The method of claim 52 or 53, wherein said method comprises administering to said smooth muscle cell a therapeutic agent.

15   55. A method of treating or preventing vascular disease in a patient comprising, preparing a smooth muscle cell, or tissue or organ comprising a smooth muscle cell, for transplant by treating said smooth muscle cell, or tissue or organ comprising a smooth muscle cell, with a therapeutic agent that increases A20 biological activity in said smooth muscle cell, or tissue

20   or organ comprising said smooth muscle cell, and transplanting said smooth muscle cell, or tissue or organ comprising said smooth muscle cell, treated with said therapeutic agent to a patient at risk for developing vascular disease.

56. A method of treating or preventing vascular disease in a patient comprising preparing a smooth muscle cell, or tissue or an organ comprising a smooth muscle cell, for transplant by treating said smooth muscle cell, or tissue, or organ comprising a smooth muscle cell, with a

5   therapeutic agent that increases the A20 level in said smooth muscle cell, or tissue or organ comprising said smooth muscle cell, and transplanting said smooth muscle cell, or tissue or organ comprising said smooth muscle cell, treated with said therapeutic agent to a patient at risk for developing vascular disease.
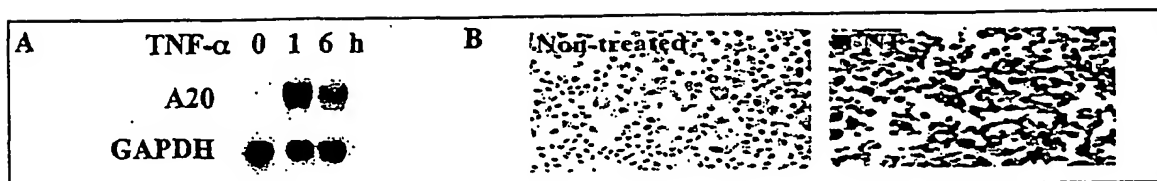
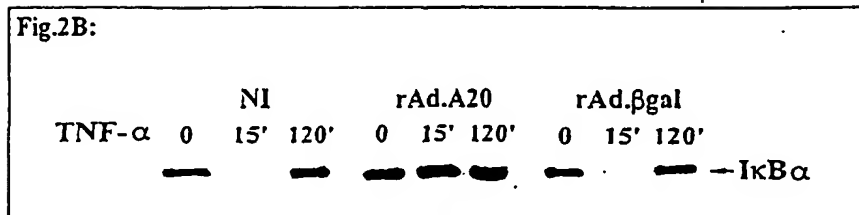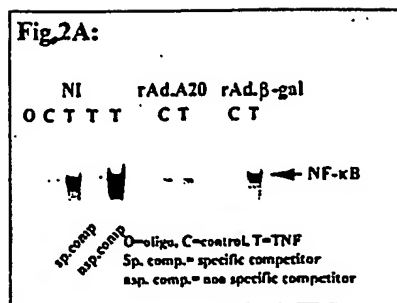Fig. 1 (A&B)

Fig.2A:

NI   rAd.A20   rAd.β-gal
O C T T T   C T   C T

← NF-κB

O=oligo, C=control T=TNF
Sp. comp.= specific competitor
nsp. comp.= non specific competitor

Fig.2B:

TNF-α   0   15' 120'   0   15' 120'   0   15' 120'
                 NI   rAd.A20   rAd.βgal

─IκBα

Fig.3:

	rAd.A20	rAd.β-gal
ICAM-1		━ ━ ━
GAPDH	━ ━ ━ ━	━ ━ ━ ━
TNF-α	0 2 4 8	0 2 4 8 h

Fig.4:

Fig.5:

Fig. 6A&B:
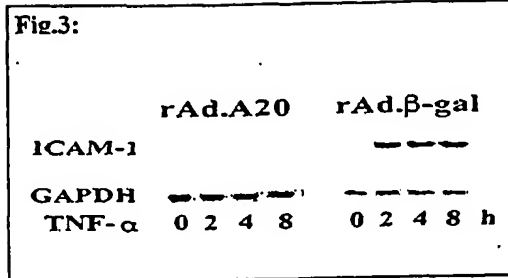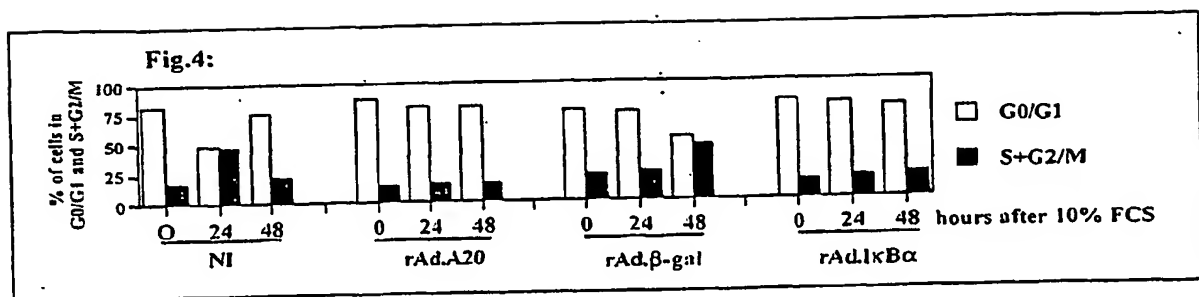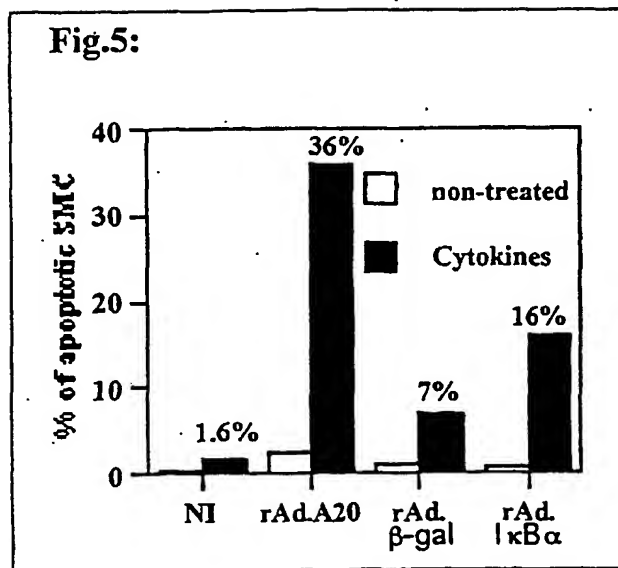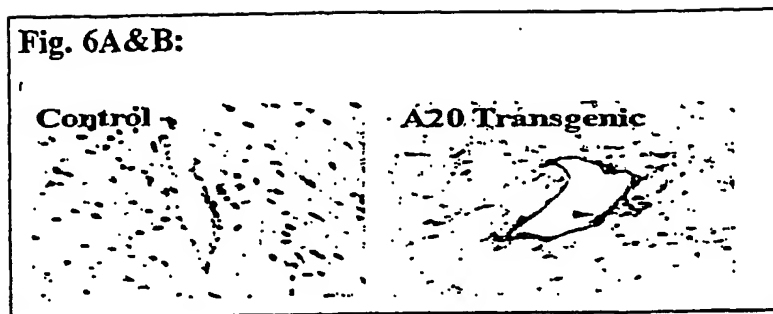
Fig. 7

Fig. 8

Fig. 9

Fig. 10

SEQUENCE LISTING

<110> Beth Israel Deaconess Medical Center

<120> Use of Pro-Apoptotic Factors in
      Treatment of Atherosclerosis

<130> 01948/067WO2

<150> US 60/177,535
<151> 2000-01-21

<160> 4

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 4426
<212> DNA
<213> Homo sapiens

<400> 1
```
tgccttgacc aggacttggg actttgcgaa aggatcgcgg ggcccggaga ggtgttggag 60
agcacaatgg ctgaacaagt ccttcctcag gctttgtatt tgagcaatat gcggaaagct 120
gtgaagatac gggagagaac tccagaagac attttaaac ctactaatgg gatcattcat 180
cattttaaaa ccatgcaccg atacacactg gaaatgttca gaacttgcca gttttgtcct 240
cagtttcggg agatcatcca caaagccctc atcgacagaa acatccaggc caccctggaa 300
agccagaaga aactcaactg gtgtcgagaa gtccggaagc ttgtggcgct gaaaacgaac 360
ggtgacggca attgcctcat gcatgccact tctcagtaca tgtgggggcgt tcaggacaca 420
gacttggtac tgaggaaggc gctgttcagc acgctcaagg aaacagacac acgcaacttt 480
aaattccgct ggcaactgga gtctctcaaa tctcaggaat ttgttgaaac ggggctttgc 540
tatgatactc ggaactggaa tgatgaatgg gacaatctta tcaaaatggc ttccacagac 600
acacccatgg cccgaagtgg acttcagtac aactcactgg aagaaataca catatttgtc 660
ctttgcaaca tcctcagaag gccaatcatt gtcatttcag acaaaatgct aagaagtttg 720
gaatcaggtt ccaatttcgc cctttgaaa gtgggtggaa tttacttgcc tctccactgg 780
cctgcccagg aatgctacag ataccccatt gttctcggct atgacagcca tcattttgta 840
cccttggtga ccctgaagga cagtgggcct gaaatccgag ctgttccact tgttaacaga 900
gaccggggaa gatttgaaga cttaaaagtt cacttttga cagatcctga aatgagatg 960
aaggagaagc tcttaaaaga gtacttaatg gtgatagaaa tccccgtcca aggctgggac 1020
catggcacaa ctcatctcat caatgccgca aagttggatg aagctaactt accaaaagaa 1080
atcaatctgg tagatgatta ctttgaactt gttcagcatg agtacaagaa atggcaggaa 1140
aacagcgagc aggggaggag agaggggcac gcccagaatc ccatggaacc ttccgtgccc 1200
cagctttctc tcatggatgt aaaatgtgaa acgcccaact gccccttctt catgtctgtg 1260
aacacccagc ctttatgcca tgagtgctca gagaggcggc aaaagaatca aaacaaactc 1320
ccaaagctga actccaagcc gggccctgag gggctccctg gcatggcgct cggggcctct 1380
cggggagaag cctatgagcc cttggcgtgg aaccctgagg agtccactgg ggggcctcat 1440
tcggccccac cgacagcacc cagcccttt ctgttcagtg agaccactgc catgaagtgc 1500
aggagccccg gctgccccctt cacactgaat gtgcagcaca acggatttg tgaacgttgc 1560
cacaacgccc ggcaacttca cgccagccac gccccagacc acacaaggca cttggatccc 1620
gggaagtgcc aagcctgcct ccaggatgtt accaggacat ttaatgggat ctgcagtact 1680
tgcttcaaaa ggactacagc agaggcctcc tccagcctca gcaccagcct ccctccttcc 1740
tgtcaccagc gttccaagtc agatccctcg cggctcgtcc ggagcccctc ccgcattct 1800
tgccacagag ctggaaacga cgcccctgct ggctgcctgt ctcaagctgc acggactcct 1860
ggggacagga cggggacgag caagtgcaga aagccggct gcgtgtattt tgggactcca 1920
gaaaacaagg gcttttgcac actgtgtttc atcgagtaca gagaaaacaa acattttgct 1980
gctgcctcag ggaaagtcag tcccacagcg tccaggttcc agaacaccat tccgtgcctg 2040
gggagggaat gcggcaccct tggaagcacc atgtttgaag gatactgcca gaagtgtttc 2100
attgaagctc agaatcagag atttcatgag gccaaaagga cagaagagca actgagatcg 2160
agccagcgca gagatgtgcc tcgaaccaca caaagcacct caaggcccaa gtgcgcccgg 2220
gcctcctgca gaaacatcct ggcctgccgc agcgaggagc tctgcatgga gtgtcagcat 2280
cccaaccaga ggatgggccc tgggggcccac cggggtgagc ctgcccccga agacccccccc 2340
```
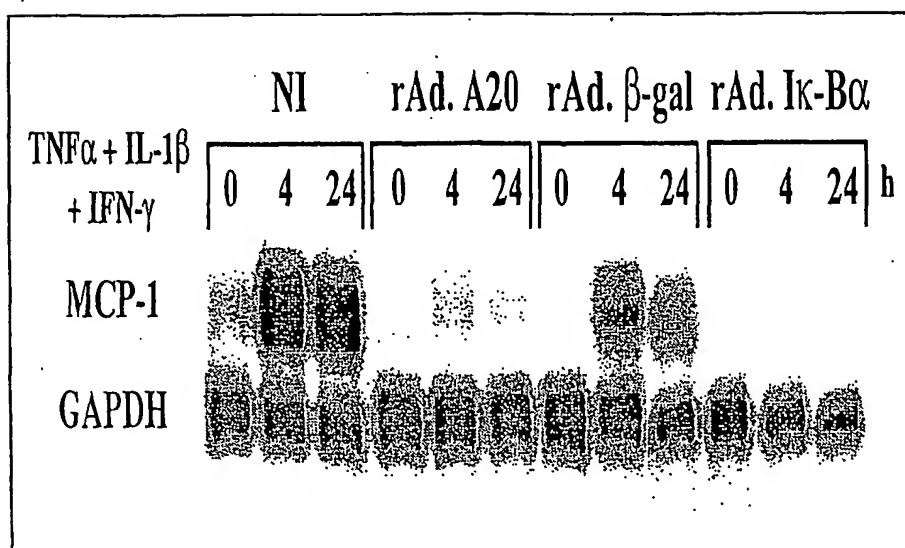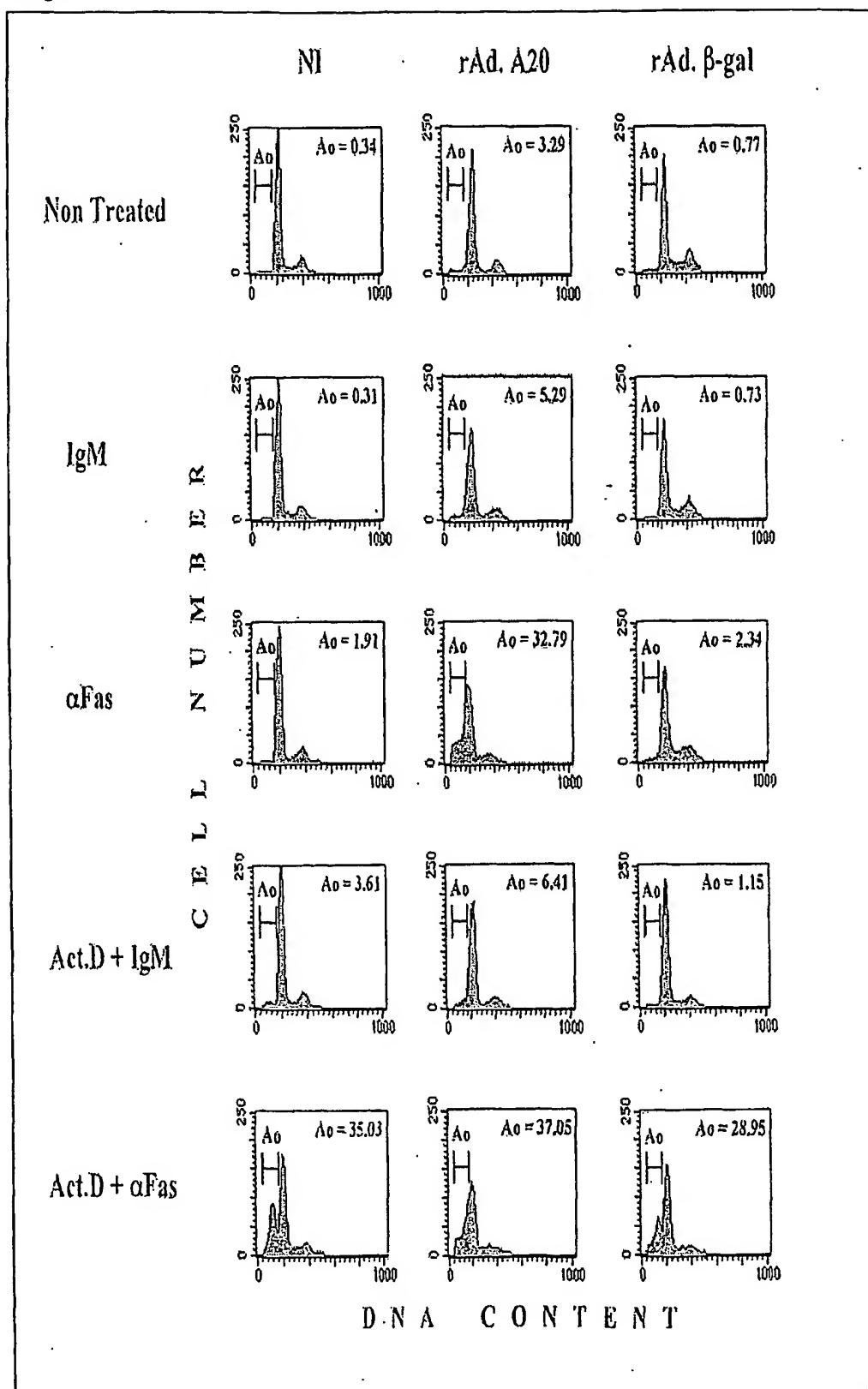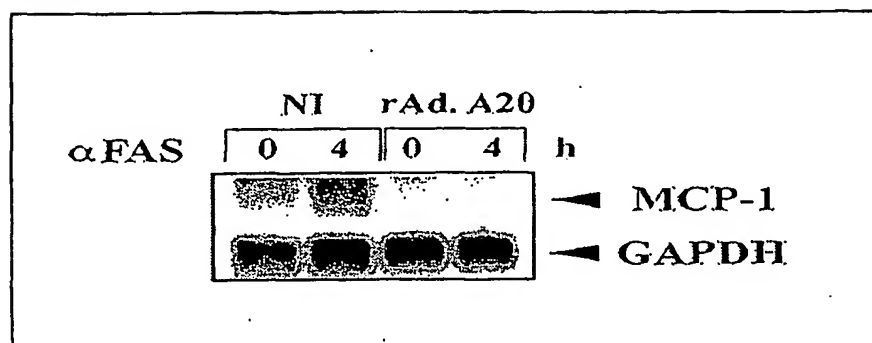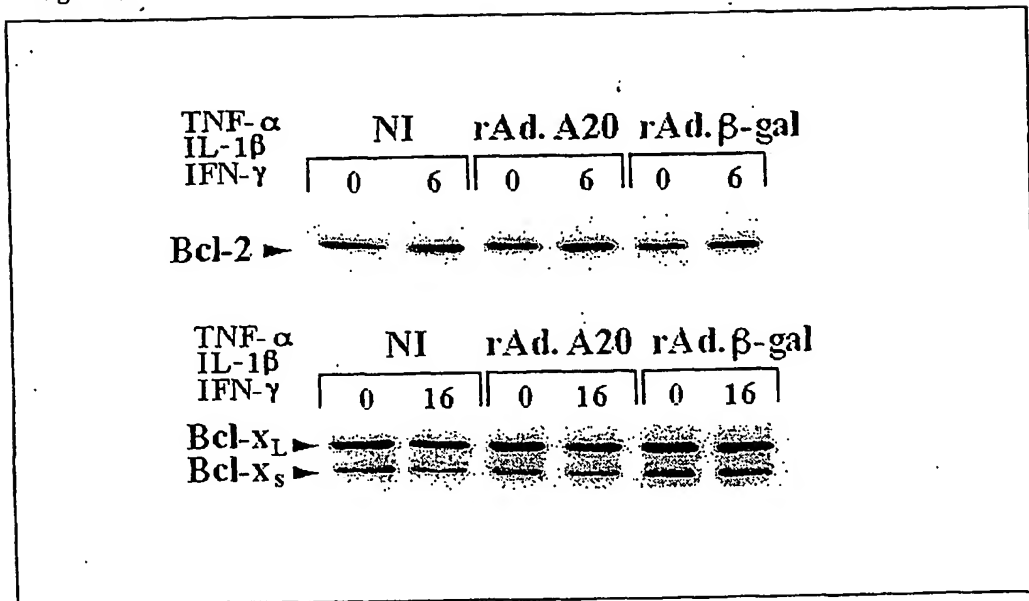
```
aagcagcgtt gccgggcccc cgcctgtgat cattttggca atgccaagtg caacggctac 2400
tgcaacgaat gctttcagtt caagcagatg tatggctaac cggaaacagg tgggtcacct 2460
cctgcaagaa gtggggcctc gagctgtcag tcatcatggt gctatcctct gaacccctca 2520
gctgccactg caacagtggg cttaagggtg tctgagcagg agaggaaaga taagctcttc 2580
gtggtgccca cgatgctcag gtttggtaac ccgggagtgt tcccaggtgg ccttagaaag 2640
caaagcttgt aactggcaag ggatgatgtc agattcagcc caaggttcct cctctcctac 2700
caagcaggag gccaggaact tctttggact tggaaggtgt gcggggactg ccgaggccc 2760
ctgcaccctg cgcatcagga ctgcttcatc gtcttggctg agaaagggaa aagacacaca 2820
agtcgcgtgg gttggagaag ccagagccat tccacctccc ctcccccagc atctctcaga 2880
gatgtgaagc cagatcctca tggcagcgag ccctctgca agaagctcaa ggaagctcag 2940
ggaaaatgga cgtattcaga gagtgtttgt agttcatggt ttttccctac ctgcccggtt 3000
cctttcctga ggacccggca gaaatgcaga accatccatg gactgtgatt ctgaggctgc 3060
tgagactgaa catgttcaca ttgacagaaa aacaagctgc tctttataat atgcaccttt 3120
taaaaaatta gaatatttta ctgggaagac gtgtaactct ttgggttatt actgtcttta 3180
cttctaaaga agttagcttg aactgaggag taaaagtgtg tacatatata atatacccct 3240
acattatgta tgagggattt ttttaaatta tattgaaatg ctgccctaga agtacaatag 3300
gaaggctaaa taataataac ctgttttctg gttgttgttg gggcatgagc ttgtgtatac 3360
actgcttgca taaactcaac cagctgcctt tttaaaggga gctctagtcc ttttttgtgta 3420
attcactttta tttattttat tacaaacttc aagattattt aagtgaagat atttcttcag 3480
ctctggggaa aatgccacag tgttctcctg agagaacatc cttgctttga gtcaggctgt 3540
gggcaagttc ctgaccacag ggagtaaatt ggcctctttg atacacttt gcttgcctcc 3600
ccaggaaaga aggaattgca tccaaggtat acatacatat tcatcgatgt ttcgtgcttc 3660
tccttatgaa actccagcta tgtaataaaa aactatactc tgtgttctgt taatgcctct 3720
gagtgtccta cctccttgga gatgagatag ggaaggagca gggatgagac tggcaatggt 3780
cacagggaaa gatgtggcct tttgtgatgg ttttatttc tgttaacact gtgtcctggg 3840
ggggctggga agtccctgc atcccatggt accctggtat tgggacagca aaagccagta 3900
accatgagta tgaggaaatc tctttctgtt gctggcttac agtttctctg tgtgctttgt 3960
ggttgctgtc atatttgctc tagaagaaaa aaaaaaagg aggggaaatg catttttccccc 4020
agagataaag gctgccattt tgggggtctg tacttatggc ctgaaaatat ttgtgatcca 4080
taactctaca cagcctttac tcatactatt aggcacactt cccccttaga gcccctaag 4140
ttttttccag acgaatcttt ataatttcct ttccaaagat accaaataaa cttcagtgtt 4200
ttcatctaat tctcttaaag ttgatatctt aatattttgt gttgatcatt atttccattc 4260
ttaatgtgaa aaaaagtaat tatttatact tattataaaa agtatttgaa atttgcacat 4320
ttaattgtcc ctaatagaaa gccacctatt ctttgttgga tttcttcaag tttttctaaa 4380
taaatgtaac ttttcacaag agtcaacatt aaaaaataaa ttattt         4426
```

<210> 2
<211> 790
<212> PRT
<213> Homo sapiens

<400> 2

```
Met Ala Glu Gln Val Leu Pro Gln Ala Leu Tyr Leu Ser Asn Met Arg
1               5                   10                  15
Lys Ala Val Lys Ile Arg Glu Arg Thr Pro Glu Asp Ile Phe Lys Pro
            20                  25                  30
Thr Asn Gly Ile Ile His His Phe Lys Thr Met His Arg Tyr Thr Leu
        35                  40                  45
Glu Met Phe Arg Thr Cys Gln Phe Cys Pro Gln Phe Arg Glu Ile Ile
    50                  55                  60
His Lys Ala Leu Ile Asp Arg Asn Ile Gln Ala Thr Leu Glu Ser Gln
65                  70                  75                  80
Lys Lys Leu Asn Trp Cys Arg Glu Val Arg Lys Leu Val Ala Leu Lys
                85                  90                  95
Thr Asn Gly Asp Gly Asn Cys Leu Met His Ala Thr Ser Gln Tyr Met
            100                 105                 110
Trp Gly Val Gln Asp Thr Asp Leu Val Leu Arg Lys Ala Leu Phe Ser
        115                 120                 125
Thr Leu Lys Glu Thr Asp Thr Arg Asn Phe Lys Phe Arg Trp Gln Leu
    130                 135                 140
Glu Ser Leu Lys Ser Gln Glu Phe Val Glu Thr Gly Leu Cys Tyr Asp
145                 150                 155                 160
```

```
Thr Arg Asn Trp Asn Asp Glu Trp Asp Asn Leu Ile Lys Met Ala Ser
            165                 170                 175
Thr Asp Thr Pro Met Ala Arg Ser Gly Leu Gln Tyr Asn Ser Leu Glu
            180                 185                 190
Glu Ile His Ile Phe Val Leu Cys Asn Ile Leu Arg Arg Pro Ile Ile
            195                 200                 205
Val Ile Ser Asp Lys Met Leu Arg Ser Leu Glu Ser Gly Ser Asn Phe
    210                 215                 220
Ala Pro Leu Lys Val Gly Gly Ile Tyr Leu Pro Leu His Trp Pro Ala
225                 230                 235                 240
Gln Glu Cys Tyr Arg Tyr Pro Ile Val Leu Gly Tyr Asp Ser His His
                245                 250                 255
Phe Val Pro Leu Val Thr Leu Lys Asp Ser Gly Pro Glu Ile Arg Ala
            260                 265                 270
Val Pro Leu Val Asn Arg Asp Arg Gly Arg Phe Glu Asp Leu Lys Val
            275                 280                 285
His Phe Leu Thr Asp Pro Glu Asn Glu Met Lys Glu Lys Leu Leu Lys
    290                 295                 300
Glu Tyr Leu Met Val Ile Glu Ile Pro Val Gln Gly Trp Asp His Gly
305                 310                 315                 320
Thr Thr His Leu Ile Asn Ala Ala Lys Leu Asp Glu Ala Asn Leu Pro
                325                 330                 335
Lys Glu Ile Asn Leu Val Asp Asp Tyr Phe Glu Leu Val Gln His Glu
            340                 345                 350
Tyr Lys Lys Trp Gln Glu Asn Ser Glu Gln Gly Arg Arg Glu Gly His
        355                 360                 365
Ala Gln Asn Pro Met Glu Pro Ser Val Pro Gln Leu Ser Leu Met Asp
    370                 375                 380
Val Lys Cys Glu Thr Pro Asn Cys Pro Phe Phe Met Ser Val Asn Thr
385                 390                 395                 400
Gln Pro Leu Cys His Glu Cys Ser Glu Arg Arg Gln Lys Asn Gln Asn
                405                 410                 415
Lys Leu Pro Lys Leu Asn Ser Lys Pro Gly Pro Glu Gly Leu Pro Gly
            420                 425                 430
Met Ala Leu Gly Ala Ser Arg Gly Glu Ala Tyr Glu Pro Leu Ala Trp
        435                 440                 445
Asn Pro Glu Glu Ser Thr Gly Gly Pro His Ser Ala Pro Pro Thr Ala
    450                 455                 460
Pro Ser Pro Phe Leu Phe Ser Glu Thr Thr Ala Met Lys Cys Arg Ser
465                 470                 475                 480
Pro Gly Cys Pro Phe Thr Leu Asn Val Gln His Asn Gly Phe Cys Glu
                485                 490                 495
Arg Cys His Asn Ala Arg Gln Leu His Ala Ser His Ala Pro Asp His
            500                 505                 510
Thr Arg His Leu Asp Pro Gly Lys Cys Gln Ala Cys Leu Gln Asp Val
        515                 520                 525
Thr Arg Thr Phe Asn Gly Ile Cys Ser Thr Cys Phe Lys Arg Thr Thr
    530                 535                 540
Ala Glu Ala Ser Ser Ser Leu Ser Thr Ser Leu Pro Pro Ser Cys His
545                 550                 555                 560
Gln Arg Ser Lys Ser Asp Pro Ser Arg Leu Val Arg Ser Pro Ser Pro
                565                 570                 575
His Ser Cys His Arg Ala Gly Asn Asp Ala Pro Ala Gly Cys Leu Ser
            580                 585                 590
Gln Ala Ala Arg Thr Pro Gly Asp Arg Thr Gly Thr Ser Lys Cys Arg
        595                 600                 605
Lys Ala Gly Cys Val Tyr Phe Gly Thr Pro Glu Asn Lys Gly Phe Cys
    610                 615                 620
Thr Leu Cys Phe Ile Glu Tyr Arg Glu Asn Lys His Phe Ala Ala Ala
625                 630                 635                 640
Ser Gly Lys Val Ser Pro Thr Ala Ser Arg Phe Gln Asn Thr Ile Pro
                645                 650                 655
```

```
Cys Leu Gly Arg Glu Cys Gly Thr Leu Gly Ser Thr Met Phe Glu Gly
        660                 665                 670
Tyr Cys Gln Lys Cys Phe Ile Glu Ala Gln Asn Gln Arg Phe His Glu
        675                 680                 685
Ala Lys Arg Thr Glu Glu Gln Leu Arg Ser Ser Gln Arg Arg Asp Val
    690                 695                 700
Pro Arg Thr Thr Gln Ser Thr Ser Arg Pro Lys Cys Ala Arg Ala Ser
705                 710                 715                 720
Cys Lys Asn Ile Leu Ala Cys Arg Ser Glu Glu Leu Cys Met Glu Cys
                725                 730                 735
Gln His Pro Asn Gln Arg Met Gly Pro Gly Ala His Arg Gly Glu Pro
            740                 745                 750
Ala Pro Glu Asp Pro Pro Lys Gln Arg Cys Arg Ala Pro Ala Cys Asp
        755                 760                 765
His Phe Gly Asn Ala Lys Cys Asn Gly Tyr Cys Asn Glu Cys Phe Gln
    770                 775                 780
Phe Lys Gln Met Tyr Gly
785             790
```

```
<210> 3
<211> 10
<212> DNA
<213> Homo sapiens

<400> 3
ggaaatcccc                                              10

<210> 4
<211> 10
<212> DNA
<213> Homo sapiens

<400> 4
ggaaagtccc                                              10
```

-4-

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/01894

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A01K 61/00, 63/00, 1/00; C12N 5/00, 5/02, 15/00
US CL : 514/44; 424/93.1; 435.1.1,325; 800/8,21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/44; 424/93.1; 435.1.1,325; 800/8,21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAPLUS, MEDLINE, USPATFUL

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 6,043,055 A (HSUEH et al) 28 mar 2000 (28.03.2000) abstract, columns 10 −13.	1-32 and 35-54
A,P	KIM TAE-HYOUNG et al. Bid-induced cytochrome c release is mediated by a pathway independent of mitochondrial permeability transition pore and Bax. Proceedings of National Academy of Sciences, USA. 15 December 2000, Vol. 276, No. 50, pages 39474-39481, see the entire document.	1-32 and 35-54
A,P	Database CAPLUS on STN. GUO B. et al. "Overexpression of Bax enhances antitumor activity of chemotherapeutic agents in human head and neck squamous cell carcinoma." Accession Number 2000:162761. Clinical Cancer Research. 2000, Vol. 6, No. 2, pages 718-724, see Abstract.	1-32 and 35-54

☐ Further documents are listed in the continuation of Box C.	☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier application or patent published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 April 2001 (09.04.2001)	08 JUN 2001
Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Ram R. Shukla
Facsimile No. (703)305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/01894

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-32 and 35-54

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-32 and 35-54, drawn to an organ of a transgenic mammal comprising a DNA encoding a pro-apoptotic protein that has A20 activity, a method of treatment using the tissue and cells expressing a DNA encoding a pro-apoptotic protein that has A20 activity.

Group II, claim(s) 33-34, drawn to a method of identifying compounds that promote apoptosis by modifying the apoptosis promoting activity of A20.

Group III, claim(s) 55, drawn to a method of treatment by increasing A20 biological activity using a therapeutic agent that is not a wild type A20 encoding DNA itself.

Group IV, claim(s) 56, drawn to a method of treatment by increasing the level of A20 using a therapeutic agentthat is not a wild type A20 encoding DNA itself.

The inventions listed as Groups I-VI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The inventions of groups I-IVI all related to methods and compositions that encompass a pro-apoptotic protein that has A20 activity. However, practicing the methods of groups II-IV would depend on compositions other than A20 encoding DNA itself and therefore lack the same special technical feature as the method of group I. For example, the method of group II will require steps that are for screening of compounds, compared to the method steps of group I which are for treatment. It is noted that a method for screening compounds that promote apoptosis can not be used for treating a patient. Likewise, the steps of a method of increasing biological activity of A20 using a therapeutic agent would lack the same special technical feature as a method of increasing the level of A20 using a therapeutic agent because a compound that would increase the amount of A20 protein would not alter the biological activity of A20 because such would require interaction of the therapeutic agent with one or more functional motifs of the A20 protein. On the other hand, a therapeutic agent that increases the amount of A20 protein may do so by altering the transcriptional regulation of the promoter driving the expression of the A20 coding DNA sequences or by increasing the translation efficiency of the A20 mRNA transcripts. Threfore, the methods of the groups I, III, and IV lack the same special technical feature.